CHEMOPREVENTIVE AND ANTIOXIDANT ACTIVITY OF *CICHORIUM INTYBUS* LEAVES AGAINST NDEA INDUCED HEPATOCARCINOGENESIS IN WISTAR RATS

Dissertation submitted to

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MASTER OF PHARMACY IN

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Submitted By

Reg.No.26113092

Under The Guidance Of

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OCTOBER – 2013

EVALUATION CERTIFICATE

This is to certify that the dissertation work entitled **"Chemopreventive and antioxidant activity of *Cichorium Intybus* Leaves against NDEA Induced Hepatocarcinogenesis in Wistar rats"** submitted by the student bearing [**Reg. No: 26113092]** to "**The Tamil Nadu Dr. M.G.R. Medical University**", Chennai, in partial fulfillment for the award of Degree of **Master of Pharmacy** in **Pharmacology** was evaluated by us during the examination held on……..…..…….

Internal Examiner **External Examiner**

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Chemopreventive and antioxidant activity of *Cichorium Intybus* Leaves against NDEA Induced Hepatocarcinogenesis in Wistar rats"** submitted to "**The Tamil Nadu Dr. M.G.R. Medical University**", Chennai, in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Miss. E. Krishnaveni, [Reg.No.26113092]** during the academic year 2012-2013, under the guidance and supervision of **Mr.V.Rajesh, M.Pharm., Ph.D.,** Assistant professor and Head, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

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DECLARATION

I do here by declared that the dissertation entitled **"Chemopreventive and antioxidant activity of *Cichorium Intybus* Leaves against NDEA Induced Hepatocarcinogenesis in Wistar rats"** submitted to "**The Tamil Nadu Dr. M.G.R Medical University"**, Chennai, for the partial fulfillment of the degree of **Master of Pharmacy** in **Pharmacology,** is a bonafide research work has been carried out by me during the academic year 2012-2013, under the guidance and supervision of **Mr. V. Rajesh, M.Pharm., Ph.D.,** Assistant professor & Head, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy , Komarapalayam .

I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma, associate ship and fellowship or any other similar title. The information furnished in this dissertation is genuine to the best of my knowledge.

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Date: **Reg. No.26113092.**

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E. Krishnaveni

Reg.No:26113092

Animal Ethical Committee Clearance Certificate

We, the Undersigned Chairman/Members of the Animal Ethical Committee, functioning in JKK Nattraja College of Pharmacy have studied the proposed research Subject/Project of **E. Krishanveni** titled **"Chemopreventive and Antioxidant Activity of *Cichorium intybus* Leaves against NDEA induced Hepatocarcinogenesis In Wistar Rats"** applying for permission for animal usage and hereby give the certificate of clearance of approval by this Ethical Committee.

Signature of the Chairman/ Members of the Animal Ethical Committee

Name of the Institution:

Station :

Date :

Seal :

TABLE OF CONTENTS

CHAPTER NO.	TITLE	PAGE NO.
1	**INTRODUCTION**	1
2	**REVIEW OF LITERATURE**	34
3	**AIM AND OBJECTIVE OF WORK**	44
4	**PLAN OF WORK**	47
5	**MATERIALS AND METHODS**	48
6	**RESULTS AND DISCUSSION**	66
7	**CONCLUSION**	108
8	**BIBLOGRAPHY**	109

LIST OF ABBREVIATIONS USED

TB	Total bilirubin
FNH	Focal nodular hyperplasia
HCC	Hepatocellular cancer
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NASH	Non-alcoholic steatohepatitis
Gly	Glycyrrhizin
VLB	Vinblastin
VCR	Vincristin
VDS	Vindesine
VRLB	Vinorelbine
NSCLC	Non-small cell lung cancer
HHT	Harringtonine and homoharringtonine
CHR	Complete hematologic remission
CML	Chronic myelogenous leukemia
CA4	Combretastatin A-4 phosphate
CdK	Cyclin- dependent kinases
NDEA	N- nitroso diethylamine
ROS	Reactive oxygen species
DPPH	2,2-diphenyl-1-picryl hydrazine
$ONOO^-$	Peroxy nitrite anion

CUPRAC	Cupric reducing anti oxidant capacity
AST	Aspartate amino transferase
ALT	Alanine amino transferase
ALP	Alkaline phosphatase
GGT	Gama glutamyl transferase
H2O2	Hydrogen peroxide
AFP	Alfa feto protein
CEA	Carcino embryonic antigen
TPC	Total phenolic content
TFC	Total flavanoid content
TTC	Total tannin content
TAC	Total antioxidant capacity
CAT	Catalase
SOD	Super oxide dismutase
GSH	Reduced glutathione
LPO	Lipid peroxidation
GPX	Glutathione peroxidise
TP	Total protein
DNA	De-oxy ribonucleic acid
GAE	Gallic acid
GSH-Px	Reduced glutathione Peroxide
CMC	Carboxy Methyl Cellulose
PB	Phenobarbital
SB	Serum bilirubin
ELISA	Enzyme Linked Immnuosorbent Assay

MDA	Malondialdehyde

INTRODUCTION

LIVER

The liver, the largest organ in the body, predominantly functions as a biochemical laboratory where metabolism takes place. It has both endocrine and exocrine functions; and is also involved in numerous metabolic activities and acting as a storage depot. Once the nutritional substances and other chemicals such as drugs, carcinogens etc reach in liver, they are metabolized by hepatic enzymes. The organ is located on the right side of the abdomen just beneath the diaphragm in human. Liver is a solid organ consisting of several lobes. Each lobe is constituted with numerous lobules which are in general hexagonal in shape (Figure 1). The center of each lobule is occupied by the central vein and the periphery of the lobule is delineated by a close arrangement of hepatic artery, portal vein, and bile duct; called "portal triads". The portal triads appear at the vertices of the hexagonal lobules. The vessels generated from the portal triads ramify and distribute along the sides of the lobule, and open into the sinusoids which have thin epithelial lining, a discontinuous layer of fenestrated endothelial cells. The liver has different types of cells. Oval cells are generally found near the portal triad. This rare cell-type has been claimed as hepatic stem cells by some researchers (Zamule *et al*., 2011). However, the major cell-type in liver is the polygonal hepatic parenchymal cells (hepatocytes). Hepatic lobules are made up of more than 80% hepatocytes which have an average size of 25 μ and occupy 70-90% of liver mass, depending on the species. They have clear cell membrane; sometimes with two nuclei. They have large deposits of glycogen, often with lipid droplets and basophilic materials. They

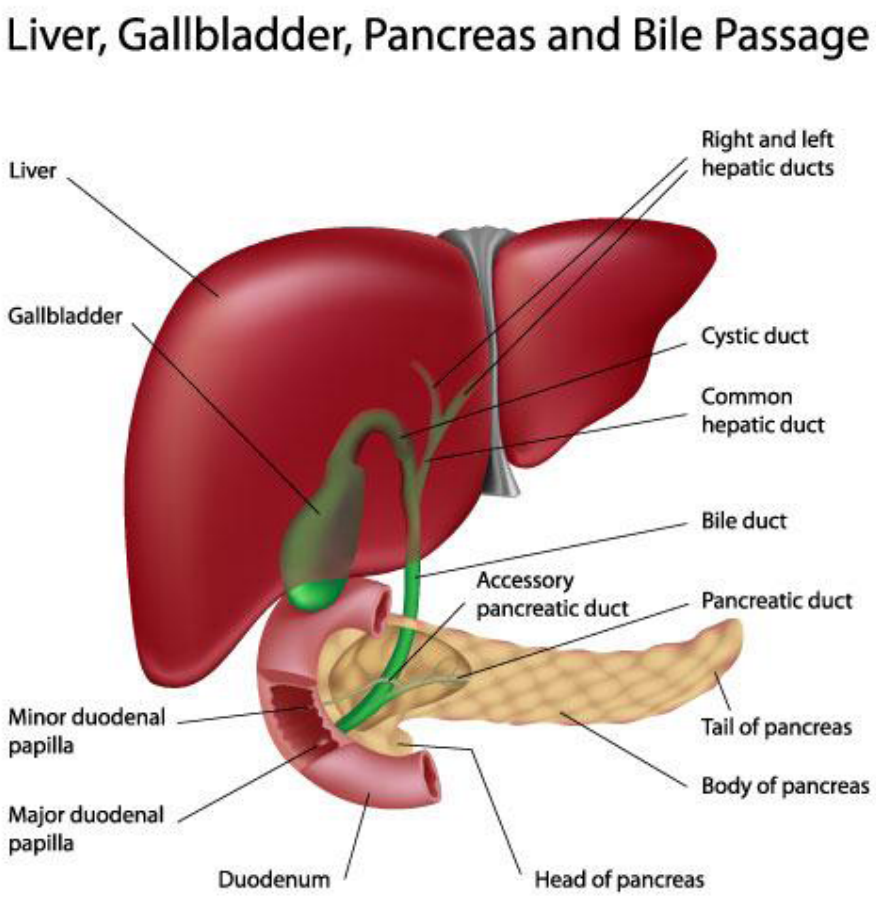
also contain other cellular organelles such as mitochondria, rough endoplasmic reticulum (granular) and smooth endoplasmic reticulum (agranular), golgi apparatus, and lysosomes. The hepatocytes are arranged in stacks of anastomosing plates, separated by an anastomosing system of sinusoids. One or two cells thick hepatocytes appear radiating from central vein towards the periphery. They metabolize and excrete into sinusoids or bile canaliculi. They can undergo cell division to produce more hepatocytes. Other than the endothelial cells, the liver sinusoids contain phagocytic cells derived from monocytes, known as Kupffer cells. These macrophages phagocytize particulates and cell debris. Another hepatic cell-type is known as the into cell. These are adipose or stellate cells.

Liver undertakes several important functions in our body.

- Produces bile which contains bile salts (sodium glycocholate, sodium taurocholate). The bile salts emulsify fats and oils and thus help in the digestion of them.
- Involves in carbohydrate and fat metabolism, hemoglobin metabolism and lipid synthesis.
- Stores many chemicals such as glycogens, vitamins, minerals and several metabolites involves in detoxification and removal of many toxic chemicals, including drugs.
- Carcinogens, and various toxins through bile from the body.
- Converts circulating ammonia into urea by urea cycle (Ornithine cycle) and thereby reduces ammonia level in blood.

- Produces serum proteins such as albumin, clotting factors.

Figure. 1



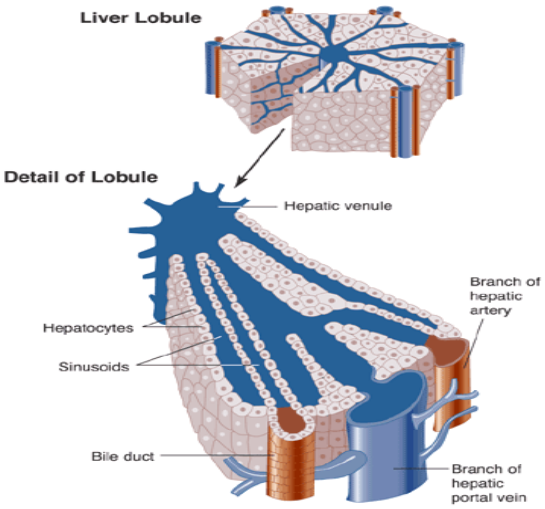
Hepatic regenerative capability

Under normal condition in an adult, liver maintains its size. However, liver has the regenerative capacity. Under various stress conditions, liver-size may increase. Numerical increase in hepatic cells by rapid cell division (hyperplasia) or increase in cell-size (hypertrophy) primarily causes enlargement of the tissue (Michalopoulos & DeFrances, 1997). Exposure to high levels of chemical toxicants causes increase in liver-size about 2-3 times of its normal size to combat the enhanced metabolic pressure exerted by chemical exposure (Michalopoulos &

DeFrances, 1997). By regeneration process liver replaces the necrotic/ dead cells or the cells damaged due to toxicity. During hepatic continual regeneration process, the increased collagen synthesis and deposition result in fibrosis. This along with further continual hepatic cell damage due to various stress conditions cause liver cirrhosis. In cirrhosis, liver morphology alters. Scarring and nodularity appear. Normal hepatic function suffers and hepatic homeostasis decreases.

Microanatomy of liver

Figure. 2



Blood drains from the sinusoids into central or centrilobular veins. These then join with veins from other lobules, forming larger veins, until eventually they become the hepatic veins, which leave the liver and empty in to the inferior vena cava. One of the functions of the liver is to secrete bile. It is seen that bile canaliculi run between the columns of liver cells. This means that each column of hepatocytes has a blood sinusoid on one side and a bile canaliculus on the other. The canaliculi join up to from larger bile canals until eventually they form the right and left hepatic ducts, which drain bile from the liver.

Lymphoid tissue and a system of lymph vessels are also present in each lobule.

Histology

The liver is divided into many hepatic lobules. Inflow to the liver involves hepatic arteries, which bring oxygenated blood to hepatic tissue, and portal veins, which bring nutrients and other compounds absorbed by the GI tract to be processed and/or stored in the liver. Outflow also volves two routes – hepatic veins which drain into the inferior vena cava and the common hepatic duct which joins the cystic duct and empties bile into the duodenum.

Major characteristics of the liver are portal triads (labeled "portal" in bottom left and shown in the middle) and central veins (labeled in bottom left and shown in the right).

The portal triad contains 1) the portal vein, 2) the hepatic artery, and 3) the bile duct. Each has its typical appearance. The central vein is lined with endothelial cells, with perforations into which the sinusoids empty.

Figure. 3

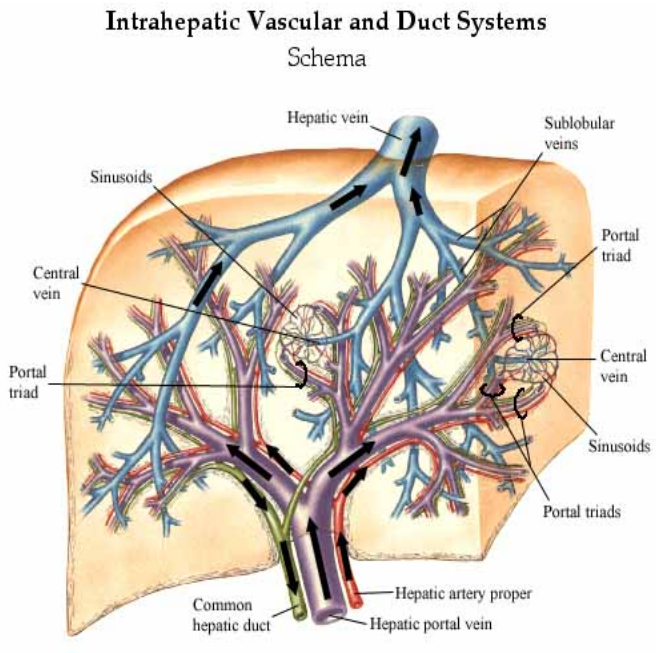
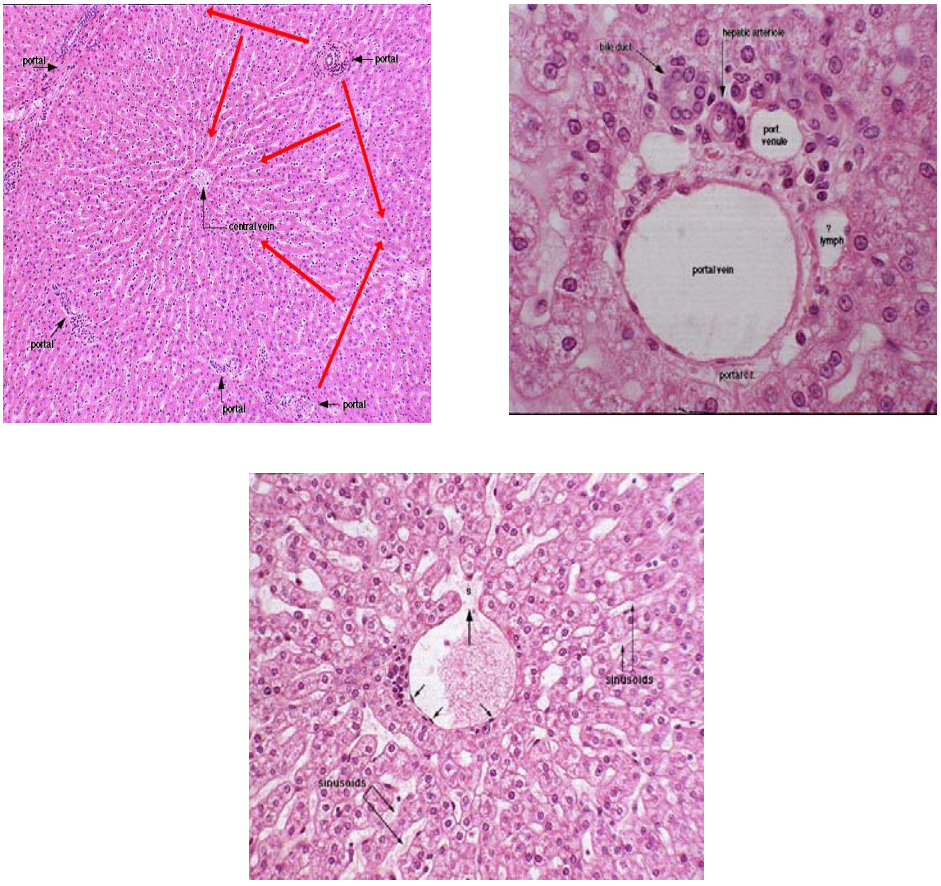


Figure. 4

Histology of normal liver



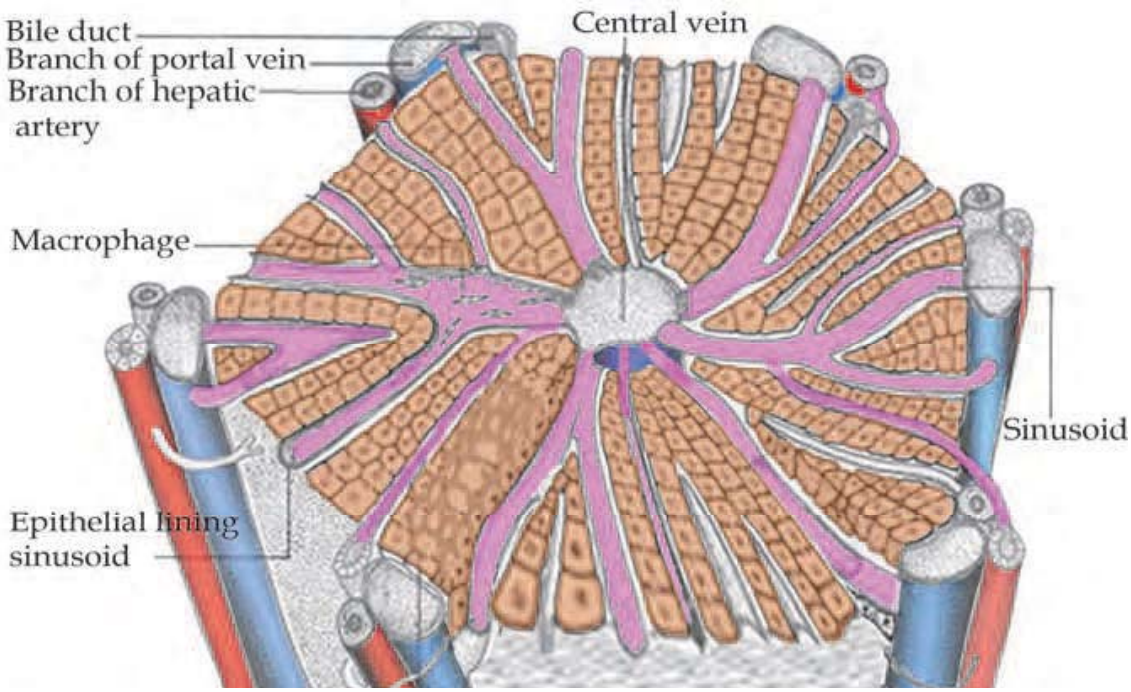
The central veins lead to sub lobular veins, which reach collecting veins, hepatic veins, and finally the inferior vena cava. The venous outflow of the liver has no regard to the organization of the lobules.

The liver sinusoids are dilated, capillary-like vessels lined by fenestrated, discontinuous epithelium (labeled “e”). Interspersed among the endothelial cells are

Kupffer cells (labeled “k”), which are fixed macrophages within the hepatic tissue. They have distinct cytoplasm that may enter the sinusoidal lumen and function like other macrophages within the body. They also break down damaged red blood cell hemoglobin. (http//www.med.unichedu.com)

There are many spaces between the hepatocytes and sinusoidal epithelial cells. They are referred to the space of Disse where exchange between hepatocytes and blood flow takes place.

Figure. 5 Histological structure of liver lobule



Liver cancer

Cancer

The body is made up of trillions of living cells. Normal body cells grow, divide, and die in anorderly fashion. During the early years of a person's life, normal

cells divide faster to allow the person to grow. After the person becomes an adult, most cells divide only to replace

worn-out or dying cells or to repair injuries. Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells. Cancer cell growth is different from normal cell growth. Instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells can also invade (grow into) other tissues, something that normal cells cannot do. Growing out of control and invading other tissues are what makes a cell a cancer cell. Cells become cancer cells because of damage to DNA. DNA is in every cell and directs all its actions. In a normal cell, when DNA gets damaged the cell either repairs the damage or the cell dies. In cancer cells, the damaged DNA is not repaired, but the cell doesn't die like it should. Instead, this cell goes on making new cells that the body does not need. These new cells will all have the same damaged DNA as the first cell does. People can inherit damaged DNA, but most DNA damage is caused by mistakes that happen while the normal cell is reproducing or by something in our environment. Sometimes the cause of the DNA damage is something obvious, like cigarette smoking. But often no clear cause is found. In most cases the cancer cells form a tumor. Some cancers, like leukemia, rarely form tumors. Instead, these cancer cells involve the blood and blood-forming organs and circulate through other tissues where they grow. Cancer cells often travel to other parts of the body, where they begin to grow and form new tumors that replace normal tissue. This process is called metastasis. It happens when the cancer cells get into the bloodstream or lymph vessels of our body. No matter where a cancer may spread, it is always named for the place where it started. For

example, breast cancer that has spread to the liver is still called breast cancer, not liver cancer. Likewise, prostate cancer that has spread to the bone is metastatic prostate cancer, not bone cancer. Different types of cancer can behave very differently. For example, lung cancer and breast cancer are very different diseases. They grow at different rates and respond to different treatments. That is why people with cancer need treatment that is aimed at their particular kind of cancer. Not all tumors are cancerous. Tumors that aren't cancer are called benign. Benign tumors can cause problems – they can grow very large and press on healthy organs and tissues. But they cannot grow into (invade) other tissues. Because they can't invade, they also can't spread to other parts of the body (metastasize). These tumors are almost never life threatening.

Liver cancer

Liver cancer is a cancer that starts in the liver. These different types of cells in the liver can form several types of malignant (cancerous) and benign (non-cancerous) tumors. These tumors have different causes, are treated differently, and have a different prognosis (www.cancer.org/cancer/livercancer).

Benign liver tumors

Benign tumors sometimes grow large enough to cause problems, but they do not grow intonearby tissues or spread to distant parts of the body. If they need to be treated, the patient can usually be cured with surgery.

Hemangioma

The most common type of benign liver tumor, hemangiomas start in blood vessels. Most hemangiomas of the liver cause no symptoms and do not need treatment. But some may bleed and need to be removed surgically.

Hepatic adenoma

Hepatic adenoma is a benign tumor that starts from hepatocytes (the main type of liver cell). Most cause no symptoms and do not need treatment. But some eventually cause symptoms, such as pain or a mass in the abdomen (stomach area) or blood loss. Because there is a risk that the tumor could rupture (leading to severe blood loss) and a small risk that it could eventually develop into liver cancer, most experts usually advise surgery to remove the tumor if possible. The use of certain drugs may increase the risk of getting these tumors. Women have a higher chance of having one of these tumors if they take birth control pills, although this is rare. Men who use anabolic steroids may also develop these. Adenomas may shrink when the drugs are stopped.

Focal nodular hyperplasia

Focal nodular hyperplasia (FNH) is a tumor-like growth made up of several cell types (hepatocytes, bile duct cells, and connective tissue cells). Although FNH tumors are benign, it can be hard to tell them apart from true liver cancers, and doctors sometimes remove them when the diagnosis is unclear. If you have symptoms from an FNH tumor, it can be removed with surgery. Both hepatic adenomas and FNH tumors are more common in women than in men.

Cancers that start in the liver

Several types of cancer can start in the liver.

Hepatocellular carcinoma (hepatocellular cancer)

This is the most common form of liver cancer in adults. It is also sometimes called hepatoma. About 4 of 5 cancers that start in the liver are this type.

Hepatocellular cancer (HCC) can have different growth patterns:

- Some begin as a single tumor that grows larger. Only late in the disease does it spread to other parts of the liver.
- A second type seems to start as many small cancer nodules throughout the liver, not just a single tumor. This is seen most often in people with cirrhosis (chronic liver damage) and is the most common pattern seen in the United States.

Under a microscope, doctors can distinguish several subtypes of HCC. Most often these subtypes do not affect treatment or prognosis (outlook). But one of these subtypes, fibrolamellar, is important to recognize. This type is rare, making up less than 1% of HCCs.

Patients with this type are usually younger than age 35, and the rest of their liver is not diseased. This subtype generally has a better outlook than other forms of HCC.

Intrahepatic cholangiocarcinoma (bile duct cancer)

About 10% to 20% of cancers that start in the liver are intrahepatic cholangiocarcinomas. These cancers start in the cells that line the small bile ducts (tubes that carry bile to the gallbladder) within the liver. (Most cholangiocarcinomas

actually start in the bile ducts outside the liver.) Although the rest of this document deals mainly with hepatocellular cancers, cholangiocarcinomas are often treated the same way.

Angiosarcoma and hemangiosarcoma

These are rare cancers that begin in cells lining the blood vessels of the liver. People who have been exposed to vinyl chloride or to thorium dioxide (Thorotrast) are more likely to develop these cancers. See the section "What are the risk factors for liver cancer?" Some other cases are thought to be due to exposure to arsenic or radium, or to an inherited condition known as hemochromatosis. In about half of all cases, no likely cause can be identified. These tumors grow quickly and are usually too widespread to be removed surgically by the time they are found. Chemotherapy and radiation therapy may help slow the disease, but these cancers are usually very hard to treat.

Hepatoblastoma

This is a very rare kind of cancer that develops in children, usually in those younger than 4 years old. The cells of hepatoblastoma are similar to fetal liver cells. About 2 out of 3 children with these tumors are treated successfully with surgery and chemotherapy, although the tumors are harder to treat if they have spread outside of the liver.

Secondary liver cancer

Most of the time when cancer is found in the liver it did not start there but has spread (metastasized) from somewhere else in the body, such as the pancreas, colon, stomach, breast, or lung. These tumors are named and treated based on their

primary site (where they started). For example, cancer that started in the lung and spread to the liver is called lung cancer with spread to the liver, not liver cancer, and it is treated as lung cancer.

Risk factors for liver cancer

A risk factor is anything that affects your chance of getting a disease, such as cancer.

Different cancers have different risk factors. Scientists have found several risk factors that make a person more likely to develop hepatocellular carcinoma (HCC).

Gender

Hepatocellular carcinoma is much more common in males than in females. Much of this is probably because of behaviors affecting some of the risk factors described below. The fibrolamellar subtype of HCC occurs in about equal numbers in both sexes.

Race/ethnicity

In the United States, Asian Americans and Pacific Islanders have the highest rates of liver cancer, followed by American Indians/Alaska Natives and Hispanics/Latinos, African Americans, and whites.

Chronic viral hepatitis

Worldwide, the most common risk factor for liver cancer is chronic (long-term) infection with hepatitis B virus (HBV) or hepatitis C virus (HCV). These infections lead to cirrhosis of the liver (see below) and are responsible for making

liver cancer the most common cancer in many parts of the world.In the United States, infection with hepatitis C is the more common cause of HCC, while in Asia and developing countries, hepatitis B is more common. People infected with both viruses have a very high risk of developing chronic hepatitis, cirrhosis, and liver cancer. HBV and HCV can spread from person to person through sharing contaminated needles (such as in drug use), unprotected sex, or childbirth. They can also be passed on through blood transfusions, although this is very rare in the United States since the start of blood product testing for these viruses. In developing countries, children sometimes contract hepatitis B infection from prolonged contact with family members who are infected. Of the 2 viruses, infection with HBV is more likely to cause symptoms, such as a flu-like illness and a yellowing of the eyes and skin (jaundice). But most people recover completely from HBV infection within a few months. Only a very small percentage of adults become chronic carriers (and have a higher risk for liver cancer). The risk of becoming a chronic carrier is higher in infants and small children who become infected. HCV, on the other hand, is less likely to cause symptoms. But most people with HCV develop chronic infections, which are more likely to lead to liver damage or even cancer. Other viruses, such as the hepatitis A virus and hepatitis E virus, can also cause hepatitis. But people infected with these viruses do not develop chronic hepatitis or cirrhosis, and are not at an increased risk of liver cancer.

Heavy alcohol use

Alcohol abuse is a leading cause of cirrhosis in the United States, which in turn is linked with an increased risk of liver cancer.

Cirrhosis

Cirrhosis is a disease in which liver cells become damaged and are replaced by scar tissue. People with cirrhosis have an increased risk of liver cancer. Most (but not all) people who develop liver cancer already have some evidence of cirrhosis. There are several possible causes of cirrhosis. Most cases in the United States occur in people who abuse alcohol or have chronic HBV or HCV infections. Non-alcoholic fatty liver disease, a condition in which people who consume little or no alcohol develop a fatty liver, is common in obese people. People with a type of this disease known as non-alcoholic steatohepatitis (NASH) might go on to develop cirrhosis. Certain types of inherited metabolic diseases can cause problems in the liver that lead to cirrhosis. Some types of autoimmune diseases that affect the liver can also cause cirrhosis.

Obesity

Being obese (very overweight) increases the risk of developing liver cancer. This is probably because it can result in fatty liver disease and cirrhosis.

Type 2 diabetes

Type 2 diabetes has been linked with an increased risk of liver cancer, usually in patients who also have other risk factors such as heavy alcohol use and/or chronic viral hepatitis. This risk may be increased because people with type 2 diabetes tend to be overweight or obese, which in turn can cause liver problems.

Inherited metabolic diseases

Certain inherited metabolic diseases can lead to cirrhosis. People with hemochromatosis absorb too much iron from their food. The iron settles in tissues throughout the body, including the liver. If enough iron builds up in the liver, it can lead to cirrhosis and liver cancer.

Other rare diseases that increase the risk of liver cancer include:

- Tyrosinemia
- Alpha1-antitrypsin deficiency
- Porphyria cutanea tarda
- Glycogen storage diseases
- Wilson disease

Aflatoxins

These cancer-causing substances are made by a fungus that contaminates peanuts, wheat, soybeans, ground nuts, corn, and rice. Storage in a moist, warm environment can lead to the growth of this fungus. Although this can occur almost anywhere in the world, it is more common in warmer and tropical countries. Developed countries such as the United States and those in Europe regulate the content of aflatoxins in foods through testing. Long-term exposure to these substances is a major risk factor for liver cancer. The risk is increased even more in people with hepatitis B or C infections.

Vinyl chloride and thorium dioxide (Thorotrast)

Exposure to these chemicals raises the risk of angiosarcoma of the liver. It also increases the risk of developing cholangiocarcinoma and hepatocellular cancer, but to a far lesser degree. Vinyl chloride is a chemical used in making some kinds of plastics. Thorotrast is a chemical that in the past was injected into some patients as part of certain x-ray tests. When the cancer-causing properties of these chemicals were recognized, steps were taken to eliminate them or minimize exposure to them. Thorotrast is no longer used, and exposure of workers to vinyl chloride is strictly regulated.

Anabolic steroids

Anabolic steroids are male hormones used by some athletes to increase their strength and muscle mass. Long-term anabolic steroid use can slightly increase the risk of hepatocellular cancer. Cortisone-like steroids, such as hydrocortisone, prednisone, and dexamethasone, do not carry this same risk.

Arsenic

Drinking water contaminated with naturally occurring arsenic, such as that from some wells, over a long period of time increases the risk of some types of liver cancer. This is more common in parts of East Asia, but it might also be a concern in some areas of the United States.

Factors with uncertain, controversial, or unproven effects on liver cancer risk

Birth control pills

In rare cases, birth control pills, also known as oral contraceptives, can cause benign tumors called hepatic adenomas. But it is not known if they increase the risk of hepatocellular cancer. Some of the studies that have looked at this issue have suggested there may be a link, but most of the studies were not of high quality and looked at types of pills that are no longer used. Current birth control pills use different types of estrogens, different estrogen doses, and different combinations of estrogens with other hormones. It is not known if the newer pills increase liver cancer risk.

Tobacco use

Some studies have found a link between smoking and liver cancer, but this has been hard to study because people who smoke are also more likely to drink alcohol. The link between smoking and liver cancer seems to be strongest among people with viral hepatitis or who drink a lot of alcohol.

Although several risk factors for hepatocellular cancer are known, exactly how these factors cause normal liver cells to become cancerous is only partially understood. Cancers develop when the DNA of cells is damaged. DNA is the chemical in each of our cells that makes up our genes – the instructions for how our cells function. We usually look like our parents because they are the source of our DNA. But DNA affects more than how we look. Some genes have instructions for controlling when cells grow, divide into new cells, and die. Genes that help cells

grow and divide are called oncogenes. Genes that slow down cell division or cause cells to die at the right time are called tumor suppressor genes. Cancers can be caused by DNA changes that turn on oncogenes or turn off tumor suppressor genes. Several different genes usually need to have changes for a cell to become cancerous. Certain chemicals that cause liver cancer, such as aflatoxins, are known to damage the DNA in liver cells. For example, studies have shown that aflatoxins can damage the TP53 tumor suppressor gene, which normally works to prevent cells from growing too much. Damage to the TP 53 gene can lead to increased growth of abnormal cells and formation of cancers. Infection of liver cells with hepatitis viruses can also damage DNA. These viruses have their own DNA, which carries instructions on how to infect cells and produce more viruses. In some patients, this viral DNA can insert itself into a liver cell's DNA, where it may affect the cell's genes. But scientists still don't know exactly how this might lead to cancer. Although scientists are starting to understand how liver cancer develops, much more must be learned. Liver cancer clearly has many different causes, and there are undoubtedly many different genes involved in its development. It is hoped that a more complete understanding of how liver cancers develop will help doctors find ways to better prevent and treat them.

Signs and symptoms of liver cancer

Signs and symptoms of liver cancer often do not show up until the later stages of the disease, but sometimes they may show up sooner. If you go to your doctor when you first notice symptoms, your cancer might be diagnosed earlier, when treatment is most likely to be helpful. Some of the most common symptoms of liver cancer are:

- Weight loss (without trying)
- Loss of appetite
- Feeling very full after a small meal
- Nausea or vomiting
- Fever
- An enlarged liver, felt as a mass under the ribs on the right side
- An enlarged spleen, felt as a mass under the ribs on the left side
- Pain in the abdomen or near the right shoulder blade
- Swelling or fluid build-up in the abdomen
- Itching
- Yellowing of the skin and eyes (jaundice)
- Enlarged veins on the belly that become visible through the skin.
- Many of the signs and symptoms of liver cancer can also be caused by other conditions, including other liver problems.
- Some liver tumors make hormones that act on organs other than the liver. These hormones may cause:

- High blood calcium levels (hypercalcemia), which can cause nausea, confusion, constipation, weakness, or muscle problems

- Low blood sugar levels (hypoglycemia), which can cause fatigue or fainting
- Breast enlargement (gynecomastia) and/or shrinking of the testicles in men
- High counts of red blood cells (erythrocytosis) which can cause someone to look red and flushed
- High cholesterol levels. (www.cancer.org/cancer/livercancer)

Herbal medicine

Herbal medicine is still the mainstay of about 75 - 80% of the world population, mainly in the developing countries, for primary health care (Kamboj, 2000). This is primarily because of the general belief that herbal drugs are without any side effects besides being cheap and locally available (Gupta and Raina, 1998). According to the World Health Organization (WHO), the use of herbal remedies throughout the world exceeds that of the conventional drugs by two to three times. The use of plants for healing purposes predates human history and forms the origin of much modern medicine. Many conventional drugs originated from plant sources: a century ago, most of the few effective drugs were plant based. Examples include aspirin (willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Vickers and Zollman, 1999).

Medicinal Plants with anticancer properties

The isolation of the vinca alkaloids, vinblastine (**1**) and vincristine (**2**) from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apo-cynaceae) introduced a new era of the use of plant material as anticancer agents. They were the first agents to advance into clinical use for the treatment of cancer (Cragg and

Newman, 2005). Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma (Cragg and Newman, 2005).

Camptotheca acuminate

Camptotheca acuminata is a Chinese ornamental tree belonging to the family Nyssaceae. Camptothecin is an alkaloid that possess anticancer activity and has been extracted from the stem wood of *Camptotheca acuminate*, also known as 'tree of joy'. The first generation analogues of Camptothecin is hycamptin and camptosar that are used for the treatment of ovarian and colon cancers (Saltz *et al*., 2000). The major disadvantage in the use of Camptothecin analogues in clinical studies is a marked loss of therapeutic activity just due to their intrinsic instabilities that is resulting from the rapid hydrolysis of the lactone ring. Camptothecin is a member of the quinoline alkaloid group. Camptothecin occurs in different plant parts like the roots, twigs and leaves. It also consists of pentacyclic ring structure. The pentacyclic ring structure is to be essential for the anticancer activity but recently it has been reported that it is not essential for its activity. Camptothecin is selectively cytotoxic to S-phase cells, it arrests cells in the G-2 phase and induces fragmentation of chromosomal DNA.It shows anticancer activity mainly for solid tumours. comptothecin inhibits DNA topoisomerase1 (Redinbo *et al*., 1998). It shows anticancer activity mainly against colon and pancreatic cancer cells. But the analogues of comptothecin shows anticancer activity in breast, liver and prostate etc. Camptothecin was approved by the US Food and Drug administration in the 1970s against colon carcinoma (Moertel *et al*., 1972).

Taxus brevifolia

Taxus is a small *coniferous* trees or shrubs belonging to the family Taxaceae. The various species of Taxus has been found to possess anticancer agent i.e. Taxol (Generic name Paclitaxel and trade name Taxol). Taxol is a complex polyoxygenated diterpenoid isolated from the pacific yew, *Taxus brevifolia*. It was discovered in the late 1960s as an antineoplastic agent. Later on, it was isolated from several other species of Taxus including Taxus wallichiana.the Himalayan yew. So far, more than 300 taxoids have been isolated and characterized from different species of Taxus . Taxol as a drug has been developed by the National Cancer Institute USA. It has been used for the treatment of refractory ovarian cancer, metastatic breast and lung cancer and Kaposi's sarcoma. It has a basic pentadecane, tetracyclic ring system. It has an N-benzoyl-B-phenylisoserine side chain and this side chain is essentially required for anticancer activity. Taxol exhibits a unique mode of action. It acts as a microtubulin stabilizing agent while the other anticancer agents destabilize this process. Taxol is also referred as 'spindle poison (Wani *et al*., 1971)

Combretum caffrum

Combretum caffrum is a South African tree belonfing to the family combretaceae. The bark of this plant possesses anticancer agent i.e. Combrestatins. They are mitotic agents and have been isolated from its bark. It is found to be a potent cytotoxic agent which strongly inhibits the polymerization of brain tubulin by binding to the colchicines site. CA-4 is the most potent cytotoxic agent that possess cytotoxic activity against a wide variety of human cancer cell. CA-4 is thus an

attractive lead molecule for the development of anticancer drugs. Pettit *et al*., 1985 reported the isolation and structure of Combrestatin from the bark of African willow tree *Combretum caffrum*. Chemically, they are stilbene derivatives having two phenyl rings separated by a C-C double bond. Trimethoxy benzene moiety is essential for its activity. The compound is active against colon, lung and leukemia cancers. It is stated about this molecule that it is the most cytotoxic phytomolecule isolated so far. In vitro studies have shown that CA-4 competes with colchicines for binding sites on tubulin. Hence; it is a member of the colchicines-like inhibitors of microtubulin assembly than vinca-alkaloid type compound. It is one of a new class of anticancer agent that acts by attacking a tumor's blood supply (Chaplin *et al*., 1999). CA-4 has shown a new mode of action by targeting on vascular system. The main problem arise with this class of compounds is its poor water solubility.

Podophyllum peltatum Linnaeus

Podophyllum peltatum Linnaeus also known as American podophyllum, commonly known as the American mandrake or may apple is a North American plant belonging to the family combretaceae and it possess two naturally occurring aryl tetralin lignans i.e. Podophyllotoxin and deoxypodophyllotoxin and due to this they possess anticancer activity. They were first isolated by Podwyssotzki in 1880. Podophyllotoxin was isolated from several other species like P.emodi wall (Indian podophyllum) and *P.pleianthum* (Taiwanese podophyllum).It is a potent cytotoxic agent. Two of the semi synthetic derivatives of podophyllotoxin, i.e. etoposide and teniposide are currently used in frontline cancer chemotherapy against various cancers. It is an aryltetralin lignin, having a lactones ring. It is effective in the treatment of wilms tumours, various genital tumours and in non-Hodgkin and other

lymphomas and lung cancer. The attempts to use podophyllotoxins in the treatment of human neoplasia were mostly unsuccessful due to complicated side effects such as nausea, vomiting, damage of normal tissues etc. Extensive structure modifications were performed to obtain more potent and less toxic anticancer agents, which resulted in two semi synthetic glucosidic cyclic acetals of epipodophyllotoxin, etoposide and teniposide. These are the most widely used derivatives for the treatment of lymphomas, acute leukaemia, testicular cancer, ovarian, bladder, and brain cancers etc. Podophyllotoxin acts as an inhibitor of assembly of microtubules and arrests the cell cycle in metaphase (O'Dwyer *et al*., 1985)

***Saussurea lappa* Clarke**

S.lappa is a perennial herb belongs to family Compositae and it is indigenous to India and Pakistan, it grows in the Himalayas at 2500-3500 m elevations. The dried roots of S.lappa, called costus roots are used in the traditional system of medicine for the treatment of cancer. Sesquiterpene lactones are the most common constituents of *S. lappa*. Sesquiterpenes exert their antitumor activity by triggering apoptosis in human leukemia cells. A thorough literature survey indicated that Sesquiterpenes lactones are rich in costus roots of which (+)-costunolide (Okugawa *et al*., 1999) is the chief constituent, which has shown potent anticancer activity and has been considered as a potential candidate for various types of tumours. The compounds extracted includes B-cyclocostunolide, dihydro costunolide and dehydro costuslactone.It has been found that exo-methylene group on lactone part of the sesquiterpenes is required for eliciting cytotoxicity. It is used against colon cancer, skin cancer, breast cancer and lung cancer.

Cymbopogon flexuous

C .flexuosus belongs to family Poaceae. The essential oil from a lemon grass variety of C.flexuosus is used for its cytotoxic activity. The oil has a promising anticancer activity and causes loss in tumors cell viability by activating the apoptotic process. *C. flexuosus* oil is also thought to help with stress related disorders and has been shown to have antifungal and antimicrobial properties (Skehan P *et al*., 1990).The various constituents present in the oil are geraniol, geranyl acetate, alpha-bisabolol and isointermedeol have been individually reported for their cancer cell cytotoxicity.The limonene and borneol present in *C .flexuosus* oil are known for their immune stimulatory activity, analgesic and anesthetic activities respectively. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achieved by inducing apoptosis in cancer cells, which is achieved by using the oil of *C .flexuosus*. The oil shows significant cytotoxic activity against colon cancer, neuroblastoma, Hep-g-2(liver) and cervix cancer.

Litchi chinensis

Litchi chinensis is an exotic fruit belonging to the family sapindaceae. This plants generally found in South East Asia, especially in China. Litchi pericarp tissues comprise of significant amount of flavanoids [Zhang *et al*., 2000]. Flavanoids play important pharmacological roles against diseases such as cardiovascular diseases ,cancer, inflammation and allergy. Epicatechin,proanthocyanidin B2 and proanthocyanidin B4 fractions were isolated from the plant and possess cytotoxic activity against breast cancer. Isoflavone daidzein could stimulate murine non

specific, activate humoral immunity and enhance cell mediated immunity. A reduced risk of breast cancer incidence has been associated with a high intake of genistein and moderate consumption of red wine. Wang *et al.,* has reported the anticancer activity of Litchi fruit pericarp against human breast cancer and hepatocellular carcinoma. A number of studies have suggested that flavanoids play a protective role in preventing breast cancer. Rodgers and Grant suggested that the possible mechanisms responsible for anti-breast cancer prevention by flavanol and flavanol might involve in metabolizing enzymes that alter metabolic activation of potential carcinogens (Rodgers *et al*., 1998).

Myrica gale L.

Myrica gale L., a native plant from Canada, belongs to family Myricaceae is used in traditional medicine. *Myrica gale* L., known commonly as myrique baumier and sweet gale, is an aromatic shrub with broad geographical distribution at higher latitudes. Myrcene, limonene, alpha-phellandrene and betacaryophyllene were the major components isolated from this plant. The anticancer activities of these extracts were assessed against human lung carcinoma cell line and human colon adenocarcinoma cell line. Ethanobotanical studies indicate that the infusion of leaves and fruits of *Myrica gale* is widely used to treat stomach and cardiac disorders too. *Myrica gale* essential oil extracts shows anticancer activity against human lung carcinoma and colon adenocarcinoma (Halim *et al*., 1973). Sesquiterpenes are responsible for the activity of Myrica gale essential oil. Indeed alpha-humulene and farnesol are active against most tumor cell lines.

Platycodon grandiflorum

The roots of *Platycodon grandiflorum* which belongs to the family Campanulaceae and has been used in traditional medicine. The extracts from *Platycodon grandiflorum* have been reported to have a wide range of health benefits. In particular, in Korea, the roots grown for 4 years to treat bronchitis, asthma, pulmonary tuberculosis, diabetes and inflammatory diseases (Takagi *et al*., 1972). Some active compounds, such as triterpenoid and saponin have been identified. Its petroleum ether extract exhibits strong inhibitory activity against human cancer cell. Growth and the activity of the organic extract has been reported to be greater than that of the aqueous extract. *Platycodon grandiflorum* contains a strong polyacetylenic anticancer compound, which exhibited cytotoxicity on the human cancer cell lines.

Liquorice

Glycyrrhiza (Liquorice) consists of dried, peeled and unpeeled, roots and stolon of *Glycyrrhiza glabra* Linn., belonging to the family Leguminosae. The liquorice plant is a legume (related to beans and peas), native to southern Europe and parts of Asia. Sophora flavescens, a perennial herb, is a species of plant in the genus Sophora belongs to the family papilionaceous. It is a traditional Chinese medicine. Matrine (Mat), a component extract from Sophora flavescens Ait. Has a wide spectrum of pharmacological activities. Glycyrrhizin (Gly), a major active constituent of liquorice (*Glycyrrhiza glabra*) root has various pharmacological effects. Glycyrrhizinic acid is a glycoside and on hydrolysis yields glycyrrhetinic acid, which has a triterpenoid structure. Gly and Mat is ancillary drug used clinically

in china for protection of liver function and treatment of tumors. Combined use of Gly and Mat could offer better liver protection and anti hepato carcinogenic effects than Gly or Mat alone, and whether it could reduce the adverse effects of Glyalone by acetaminophen induced hepatotoxicity, diethylnitrosamine–induced hepatocarcinogenesis (Wan Xu-Ying *et al*., 2009, Ma *et al*., 2008, Shen *et al*., 2005).Glycyrrhizin (Gly) molecular formula: (C42H24N2O), a triterpene glycoside and a conjugative compound of enoxolone and glucuronic acid as an active component of liquorice has been used in preventation of liver cancer (Isbrucker and Burdock 2006, Kurisu *et al*., 2008, Bing *et al*., 2006, Kimura *et al*., 2008, Wen *et al*., 2006).

Plant-Derived Anti-Cancer Agents in Clinical Use

The first agents to advance into clinical use were the so-called vinca alkaloids, vinblastine (VLB) and vincristine (VCR), isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), which was used by various cultures for the treatment of diabetes. These drugs were first discovered during an investigation of the plant as a source of potential oral hypoglycemic agents. While research investigators could not confirm this activity, it was noted that extracts reduced white blood cell counts and caused bone marrow depression in rats, and subsequently it was found that the treatment of mice bearing a transplantable lymphocytic leukemia caused life extension. This led to the isolation of VLB and VCR as the active agents. More recent semi-synthetic analogues of these agents are vinorelbine (VRLB) and vindesine (VDS). These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of variety of cancers. VLB is used for the treatment of leukemias, lymphomas, advanced

testicular cancer, breast and lung cancers and Kaposi's sarcoma and VCR, in addition to lymphomas, also shows efficacy against leukemias, particularly acute lumphocytic leukemia in childhood. VRLB has shown activity against non-small-cell lung cancer and advanced breast cancer (Cragg and Newman, 2005).

Podophyllum species

The two clinically-active agents, etoposide (VM 26) and teniposide (VP 16-213), which are semi-synthetic derivatives of the natural product, epipodophyllotoxin (an isomer of podophyllotoxin) is used for the treatment of cancer. The *Podophyllum* species (Podophyllaceae), *P. peltatum* Linnaeus (commonly known as the American mandrake or Mayapple), and *P. emodii* Wallich from the Indian subcontinent, have a long history of medicinal use, including the treatment of skin cancers and warts. *P. peltatum* was used by the Penobscot Native Americans of Maine for the treatment of "cancer and interest was promoted by the observation in the 1940s that venereal warts could be cured by topical application of an alcohol extract of the dried roots (called podophyllin). The major active constituent, podophyllotoxin, was first isolated in 1880, but its correct structure was only reported in the 1950s. Many closely related podophyllotoxin-like lignans were isolated during this period, and several of them were introduced into clinical trials, only to be dropped due to lack of efficacy and unacceptable toxicity. Extensive research at Sandoz Laboratories in Switzerland in the 1960s and 1970s led to the development of etoposide and teniposide as clinically effective agents which are used in the treatment of lymphomas and bronchial and testicular cancers.

A more recent addition to the armamentarium of plant-derived chemotherapeutic agents is the class of molecules called taxanes. Paclitaxel initially was isolated from the bark of *Taxus brevifolia* Nutt. (Taxaceae). The use of various parts of *T. brevifolia* and other *Taxus* species (e.g. *T. canadensis* Marshall, *T. baccata* L.) by several Native American tribes for the treatment of some non-cancerous conditions has been reported, while the leaves of *T. baccata* are used in the traditional Asiatic Indian (Ayurvedic) medicine system, with one reported use in the treatment of "cancer". Paclitaxel, along with several key precursors (the baccatins), occurs in the leaves of various *Taxus* species, and the ready semi-synthetic conversion of the relatively abundant baccatins to paclitaxel, as well as active paclitaxel analogs, such as docetaxel (Taxotere), has provided a major, renewable natural source of this important class of drugs. Paclitaxel is used in the treatment of breast, ovarian and non-small-cell lung cancer (NSCLC), and has also shown efficacy against Kaposi sarcoma. Paclitaxel has also attracted attention in the potential treatment of multiple sclerosis, psoriasis and rheumatoid arthritis. Docetaxel is primarily used in the treatment of breast cancer and NSCLC.

Another important addition to the anti-cancer drug armamentarium is the class of clinically-active agents derived from camptothecin, which is isolated from the Chinese ornamental tree, *Camptotheca acuminata* Decne (Nyssaceae), known in China as the tree of joy. Camptothecin (as its sodium salt) was advanced to clinical trials by the NCI in the 1970s, but was dropped because of severe bladder toxicity. However, extensive research was performed by several pharmaceutical companies in a search for more

effective camptothecin derivatives, and Topotecan (Hycamtin), developed by SmithKline Beecham (now Glaxo SmithKline), and Irinotecan (CPT-11; Camptosar), originally developed by the Japanese company, Yakult Honsha, are now in clinical use. Topotecan is used for the treatment of ovarian and small-cell lung cancers, while Irinotecan is used for the treatment of colorectal cancers.

Other plant-derived agents in clinical use are homoharringtonine, isolated from the Chinese tree, *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (Cephalotaxaceae), and elliptinium, a derivative of ellipticine, isolated from species of several genera of the Apocynaceae family, including *Bleekeria vitensis* A. C. Sm., a Fijian medicinal plant with reputed anti-cancer properties. A racemic mixture of harringtonine and homoharringtonine (HHT) has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia. Purified HHT has shown efficacy against various leukemias, including some resistant to standard treatment, and has been reported to produce complete hematologic remission (CHR) in patients with late chronic phase chronic myelogenous leukemia (CML). Elliptinium is marketed in France for the treatment of breast cancer (Cragg and Newman, 2005).

Plant-Derived Anticancer Agents in Clinical Development

The flavone, flavopiridol is totally synthetic, but the basis for its novel structure is a natural product, rohitukine, isolated by chemists at Hoechst India Ltd. in the early 1990s from *Dysoxylum binectariferum* Hook. f. (Meliaceae), which is phylogenetically related to the Ayurvedic plant, *D. malabaricum* Bedd., used for rheumatoid arthritis. Rohitukine was isolated

as the constituent responsible for anti-inflammatory and immunomodulatory activity.

The combretastatins were isolated from the South African “bush willow”, *Combretum caffrum* (Eckl. & Zeyh.) Kuntze (Combretaceae). Species of the *Combretum* and *Terminalia* genera, both of which belong to the Combretaceae family, are used in African and Indian traditional medicine for the treatment of a variety of diseases, including hepatitis and malaria. Several *Terminalia* species have reportedly been used in the treatment of cancer. The combretastatins are a family of stilbenes which act as anti-angiogenic agents, causing vascular shutdown in tumors and resulting in tumor necrosis. A water-soluble analogue, combretastatin A-4 phosphate (CA4), has has shown promise in early clinical trials. Of interest is the number of combretastatin (CA4) mimics being developed. Three are in clinical trials, while 11 are in preclinical development.

An interesting agent in early clinical development is roscovitine which is derived from olomucine, originally isolated from the cotyledons of the radish, *Raphanus sativus* L. (Brassicaceae), but which is now produced synthetically. Olomucine stimulated interest as a result of its inhibition of cyclin-dependent kinases (Cdk), proteins which play a major role in cell cycle progression (Cragg and Newman, 2003).

REVIEW OF LITERATURE

PLANT PROFILE

Botanical name

Cichorium intybus L.

Scientific classification

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta - vascular plants
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Asterales
Family	:	Asteraceae
Genus	:	*Cichorium*

Species : *C. intybus L*

Vernacular name

Common chicory, Blue sailors, Succory, Coffeeweed, Cornflower, Wild chicory, Wild succory, Garden endive, Garden chicory, Endive.

Common name:

Chicory, Blue sailors, Succory, Coffee weed

- Hindi: Kasni, Hinduba
- Tamil: Kasni
- Marathi: Kachani
- Telugu: Kasini, kasini-vittulu
- Kannada: Chikory
- Urdu: Kasni, tukme-e-kasni, barg-e-kasni
- Sanskrit: Kasni

DESCRIPTION

Chicory is a bushy perennial herb with blue or lavender flowers. It is a bushy perennial plant that attains a height of 1 to 4 feet. The stem has edges having hard branches. Flowers occur either solitary on nearly leafless branches, or in clusters in leaf axils. Flower-heads are 2.5-4 cm across, with spreading ray-florets. The green bracts below the flowers are prominent. The outer lancelike bracts are spreading outwards, while the longer inner ones are upright. Leaves are oblong-lancelike, and

lower leaves are pinnately lobed. The upper leaves are entire, bract-like, stem-clasping. Root is like a tail of a cow and is fleshy having brownish color from outside and white color from inside. It has a length of 2 ½ feet and has a bitter taste. Chicory is grown for its leaves, or for the roots, which are baked, ground, and used as a coffee substitute in instant coffee. In India Chicory is found in the northwestern regions like Kashmir and Punjab and in areas of south India.

Parts used: Root, herb during blooming period.

CHEMICAL CONSTITUENTS

- The active compounds in chicory are inulin, sesquiterpene lactones, vitamins, minerals, fat, mannitol and latex. Fructans have been extracted from chicory roots (Bais and Ravishankar, 2001).
- The postulated biosynthesis of sesquiterpene lactones through the mevalonate-farnesyl diphosphate-germacradiene pathway has been confirmed by the isolation of a (+)-germacrene A synthase from chicory roots (Finke *et al*., 2002).
- Chemical constituents of *Cichorium intybus* L. were identified as alpha-amyrin, taraxerone, baurenyl acetate and beta-sitosterol (De Kraker *et al.,* 1998).
- Inulooligosaccharides (IOS) production from chicory extract was carried out using endoinulinase obtained from a new isolate, Xanthomonas oryzae No. 5 (Du *et al*., 1998).

- Twelve compounds were isolated from the root of *Cichorium intybus* including 2, 3, 4, 9-tetrahydro-1H-pyrido-(3,4-b)indole-3-carboxylic acid (Cho *et al.*, 2001).

- The bitter substances are primarily the two sesquiterpene lactones Lactucin and Lactucopicrin. Other ingredients are Aesculetin, Aesculin, Cichoriin, Umbelliferone, Scopoletin and 6.7-Dihydrocoumarin and further sesquiterpene lactones and their glycosides (He *et al.*, 2002).

Medicinal uses

The ancient Egyptians ate large amounts of chicory because it was believed that the plant could purify the blood and liver, while others have relied on the herb for its power to cure "passions of the heart." Chicory continues to be a popular herbal remedy due to its healing effects on several ailments.

Whole plant, especially root, contains volatile oils, which produce high toxicity to internal parasites. Chicory is used as a tonic in the treatment of gallstones, gastro-enteritis, sinus problems, cuts and bruises. Inulin, the dietary fiber found in chicory is a helpful ingredient in treating diabetes and constipation. Chicory is also often recommended for jaundice and spleen problems. The juice made of leaves and a tea made from the blooming plant help the release of gallstones, elimination of internal mucus and production of bile. They are also useful for gastrointestinal problems: digestive difficulties, gastritis and lack of appetite.

Root chicory contains volatile oils similar to those found in plants in the related genus *Tanacetum* which includes Tansy, and is similarly effective at

eliminating intestinal worms. All parts of the plant contain these volatile oils, with the majority of the toxic components concentrated in the plant's root.

Chicory is well known for its toxicity to internal parasites. Studies indicate that ingestion of chicory by farm animals results in reduction of worm burdens (Häring *et al.*, 2007) which has prompted its widespread use as a forage supplement. Only a few major companies are active in research, development, and production of chicory varieties and selections, most in New Zealand.

Chicory (especially the flower) is used as a folk medicine in Germany, is recorded in many books as an ancient German treatment for everyday ailments. It is variously used as a tonic and as a treatment for gallstones, gastro-enteritis, sinus problems and cuts and bruises. Chicory contains inulin (Roberfroid *et al.,* 2002), which may help humans with weight loss, constipation, improving bowel function, and general health. In rats, it may increase calcium absorption and bone mineral density.

Chicory has demonstrated antihepatotoxic potential in animal studies (Tabassum *et al.*, 2010).

Milala *et al.,* 2009 evaluated composition and properties of cichory extracts rich in fructants and polyphenols. The lyophilized extract obtained from cichory seeds was the richest in polyphenol compounds and contained over 10% of total phenolics, including 71% of dicaffeoylquinic acids, and was characterized by the highest antioxidant activity.

Helal *et al.,* 2011 evaluated effect of *Cichorium intybus* L on fatty liver induced by oxy tetracycline in albino rats .The treatment with cichory ameliorated

most of the evaluated biochemical parameters and improved the induced degenerative histopathological changes .The pretreatment with cichory before the induction of fatty liver gave some protection against factors that experimentally induced fatty liver.

Ali, 2012 evaluated antihyperglycemic effect of cichory leaves and vanadium consumption on diabetic experimental rats. Vanadium administered in combination with cichory was the most effective in controlling the altered glucose metabolism and antioxidant status in diabetes rats.

Kocsis *et al.*, 2003 evaluated effects of cichory on pancreas status of rats in experimental dislipidemia .The effects of bioactive molecule of cichory extract influenced the lipid metabolism and the redox balance of pancreatic tissue of rat in experimental dislipidemia.

Atta *et al.*, 2010 evaluated hepatoprotective effect of methanol extracts of *Zingifer officinale* and *Cichorium intybus*. Methanol extract of ginger (250 and 500 mg/kg) and cichory (250 and 500 mg/kg) given alone or mixed (1:1 wt/wt) significantly restored the carbon tetra chloride induced alterations in the biochemical and cellular constituents of blood .

Pillai and Damodhran, 2007 evaluated prevention of radiation-induced chromosome damage in mouse bonemarrow by aqueous leaf extract of *Cichorium intybus*. The administration of aqueous leaf extract of cichory to the animals showed significant reduction in micronucleus induction .The extract also possessed significant hydroxyl radical scavenging activity. The effectiveness of the drug to prevent chromosomal damage consequent to irritation along with its high solubility

in water, favour its application in accidental radiation exposures and nuclear accidents.

Jamshidzadeh *et al.,* 2006 evaluated hepatoprotective activity of *Cichorium intybus* L. leaves extract against carbon tetrachloride induced toxicity. The *Cichorium intybus* extract itself was toxic towards isolated hepatocytes in concentrations above 3.6 mg/ml .The results of the present study therefore supported the traditional believes on hepatoprotective effect of the *Cichorium intybus* extract, however, high concentrations were hepatotoxic.

Heibatollah *et al.,* 2008 evaluated hepatoprotective effect of *Cichorium intybus* on CCl_4 induced liver damage in rats. The leave extract at oral dosage of 200, 400, and 500 mg/kg exhibited significant ($p<0.05$) protective effect against CCl_4 induced hepatotoxicity.*Cichorium intybus* extract significantly suppressed mainly the increase in plasma acticvities of AST, ALT, ALP and TB concentration ,which are considered as markers of liver fuctional states. The results of this study confirmed the hepatoprotective activity effect of the hydro alcoholic extract of *Cichorium intybus.*

Naseem *et al.,* 2009 evaluated hepatoprotective effect of *Cichorium intybus* linn extract against carbon tetrachloride induced liver damage .Hepatoprotective effect ($p<0.001$) was observed by alcoholic extract while aqueous extract showed no significant effect against CCl_4 induced hepatic injury.

Najafzadeh *et al.,* 2011 evaluated factors related to liver function in serum of horse by administration of *Cichorium intybus*. Cichory does not affect concentration of ALT, AST, ALP, LDH, conjugated and total bilirubin, total protein

and albumin and uric acid in serum of horse in normal condition, but it may be benefit in pathological conditions.

Nandagopal *et al.,* 2007 evaluated phytochemical and antibacterial studies of cichory. The hexane and ethyl acetate root extracts of cichory showed pronounced inhibition than chloroform petroleum ether and water extracts. Root extracts showed more inhibitory action on *Bacillus subtilis*, *Staphylococcus aureus* and *Salmonella typhi* than *Micrococcus luteus* and *Escherichia coli*.

Nehal *et al.,* 2011 evaluated hepatoprotective effect of feeding celery leaves mixed with cichory leaves and Barley grains to hypercholesteromic rats. Feeding of diet supplemented lowered the elevated serum level of liver enzymes and blood lipids in rats. While, feeding plant combination of celery, chicory and barley at 15% concentration (5% from each) was more effective in decreased the elevating of liver enzymes (AST, ALT and ALP), lowered blood lipids.

Kim *et al.,* 2002 evaluated effects of the ethanol extract of C*ichorium intybus* on the immunotoxicity by ethanol in mice. These findings indicate that the immunotoxicity induced by ethanol is significantly restored or prevented by *Cichorium intybus* on the immunotoxicity by ethanol.

Pushparaj *et al.,* 2007 evaluated anti-diabetic effects of *Cichorium intybus* in streptozotocin-induced diabetic rats. Hypoglycaemic effects of ethanolic extract of C*ichorium intybus* were observed in an oral glucose tolerance test (OGTT) in which, a dose of 125mg of plant extract /kg body weight exhibited the most potent hypoglycaemic effect. Moreover daily administration of ethanolic extract of C*ichorium intybus* (125mg/kg) for 14 days to diabetic rats attenuated serum glucose

by 20% by, triglycerides by 91% and total cholesterol by 16%. However,there was no change in serum insulin levels, which ruled out the possibility that ethanolic extract of *Cichorium intybus* induces insulin secretion from pancreatic β-cells .

Sultana *et al.,* 1995 evaluated crude extracts of hepatoprotective plants, *Solanum nigrum* and *Cichorium intybus* inhibit free radical-mediated DNA damage. The effect was dependent on the concentration of plant extracts. However, the effect of C*ichorium intybus* was much pronounced as compared to the effect of S*olanum nigrum.* These studies suggest that the observed hepatoprotective effect of these crude plant extracts may be due to their ability to suppress the oxidative degradation of DNA in tissue debris.

***Zafar et al.,* 1998** evaluated anti-hepatotoxic effects of root and root callus extracts of C*ichorium intybus* L. The results revealed that *Cichorium intybus* root callus extracts could afford a better protection against carbon tetrachloride induced heptocellular damage as compared to the natural root extract.

Schmidt *et al.,* 2007 evaluated toxicological evaluation of a cichory root extract .There were no treatment–related toxic effects from cichory extract administered orally at 70,350, or 1000mg/kg/day.

Ahmed *et al.,* 2003 evaluated antihepatotoxic activity of seeds of *Cichorium intybus*. The methanol fraction and compound AB-IV were found to possess a potent antihepatotoxic activity comparable to the standard drug silymarin (silybon-70). The histopathological study of the liver was also carried out, where in the methanolic fraction and compound AB-IV showed almost complete normalization of the tissues as neither fatty accumulation nor necrosis was observed.

Suntar *et al.,* 2012 evaluated comparative evaluation of traditional prescriptions from *Cichorium intybus* L for wound healing. Stepwise isolation of an active component by in vivo bioassay and its mode of activity. That *Cichorium intybus* roots exerted a prominent wound healing activity and β-sitosterol was isolated as at least one of the active component responsible from this effect.

Jurgonski *et al.,* 2011 evaluated composition of cichory root, peel, seed and leaf ethanol extracts and biological properties of their non-inulin fractions. The chemical composition of the 75% ethanol extracts of root, peel, leaf and seed presented in this study indicates that different parts of cichory and its by–products might be good sources of functional compounds (inulin, cichoric acid, quercetin, glucuronide, chlorogenic acid and other caffeoylquinic acids. In connection with their ability to increase the protection against lipid peroxidation, points that cichory non-inulin fractions, rich in phenolics, extracted from leaves and root peels might be an additional factor in improving the physiological activity of inulin.

AIM AND OBJECTIVE

Liver cancer, especially hepatocellular carcinoma (HCC), is the fifth most common cancer and the third foremost cause of cancer associated death globally (Befeler and Di Bisceglie, 2002; Bishayee and Dhir, 2009). Hepatocellular carcinoma can be secondary to hepatitis B or C, cirrhosis due to alcohol consumption, liver disease due to aflatoxin toxicity, hormonal imbalance and certain metabolic diseases (Thorgeirsson and Grisham, 2002). Hepatocarcinogenesis involves initial genotoxic insult (initiation), clonal expansion from premalignant to malignant lesions (promotion) and finally tumor progression by further clonal expansion (Thorgeirsson and Grisham, 2002; Halsted *et al.,* 1996).

N-Nitrosodiethylamine (NDEA) a potent hepatocarcinogen, is known to cause perturbations in the nuclear enzymes involved in DNA repair/replication (Bhosale *et al.,* 2002) and is normally used as a carcinogen to induce liver cancer in animal models. Investigations have provided evidence that N-nitrosamines cause wide range of tumours in all animal species and these compounds are considered to be effective health hazards to man (Loeppky, 1994). The main cause for concern is that diethylnitrosamine is found in a wide variety of foods like cheese, soybean, smoked, salted and dried fish, cured meat and alcoholic beverages (Liao *et al.,* 2001). Metabolism of certain therapeutic drugs is also reported to produce diethylnitrosamine (Akintonwa, 1985). It is also found in tobacco smoke at a concentration ranging from 1 to 28 ng/cigarette and in baby bottle nipples at a level of 10 ppb (IARC, 1972). It has been reported that, on metabolic activation, it produces the pro-mutagenic products, *O*6-ethyl deoxy guanosine and *O*4 and *O*6-

ethyl deoxy thymidine in liver which are responsible for its carcinogenic effects (Verna *et al.,* 1996). It is also reported that the generation of reactive oxygen species (ROS) by NDEA causes carcinogenic effects. Oxidative stress caused by ROS has been reported in membrane lipid peroxidation, DNA damage and mutagenesis associated with various stages of tumor formation process (Parola and Robino, 2001). Human liver appears to metabolize nitrosamines in a manner similar to that of rodent liver and also exhibits considerable similarities with regard to morphology, genomic alterations and gene expression, despite their different disease etiologies (Feo *et al.,* 2000). Hence the model of NDEA-induced HCC is considered as one of the most accepted and widely used experimental models to study hepatocarcinogenesis (Hai *et al.,* 2001).

Chemoprevention offers a novel approach to control the incidence of liver cancer (Wattenberg and Estensen, 1996). A large number of chemotherapeutic agents have been identified in epidemiological and experimental studies, preclinical and clinical observations (Takcuji *et al.,* 1993; Jagadeeswaran *et al.,* 2000). However, the toxic effects produced by some of the agents have limited their extensive use. There is, therefore, a need to identify synthetic or natural compound that have significant chemopreventive potential to block initiation or arrest the progression in premalignant cells without undesirable side effects.

In recent years, there has been considerable interest in natural products with antioxidant property to protect cellular components from oxidative damage and prevent diseases. Over 50 % of anticancer drugs approved by United States Food and Drug Administration since 1960 were originated from terrestrial plants (Kim and Park 2002; Mann, 2002). Several constituents present naturally in medicinal

plants have been shown to modify critical reactions that cause inhibition of chemically induced hepatocarcinogenesis (Soni *et al.,*1997; Bhattacharya and Chattarjee, 1998) and this worldwide effort continues to discover new anticancer drugs from plants. Among the herbal resources is *Cichorium intybus* Linn.

Chicory has a long history of herbal use and is especially of great value for its tonic effects upon the liver and digestive tract. The root and the leaves are appetiser, cholagogue, depurative, digestive, diuretic, hypo-glycemic, laxative and tonic. A decoction of the root has been used in the treatment of jaundice, liver enlargement, gout and rheumatism. In Indian traditional therapy, chicory was stated to possess tonic properties (Chopra *et al.,*1996). The tonic of chicory was considered to be beneficial in the treatment of enlarged spleen and diarrhoea. Studies have shown that chicory preparations possessed potent anti-hepatotoxic activity (Chhaya and Mishra, 1997). The alcoholic extract of *Cichorium intybus* is being used to treat pyorrhea or gingival inflammation (Patel and Bhatt, 1985). Aqueous and alcoholic extracts showed anti-inflammatory activity against formalin-induced oedema. It is also reported to have quinidine-like action on isolated heart (Jindal *et al.,*1975). A decoction of the leaves has been used in the treatment of jaundice, liver enlargement, gout and rheumatism (Pushparaj *et al.,* 2007). Based on the widespread use of *Cichorium intybus* leaves in liver disorders, a study was planned to evaluate the chemopreventive effect of *Cichorium Intybus* leaves against NDEA induced hepatocarcinogenesis in wistar rats.

PLAN OF WORK

The objective of the present study is

i. To collect *Cichorium intybus* plant material and authenticate the plant material.

ii. To prepare extract of *Cichorium intybus* leaves using solvents of varying polarity.

iii. To determine the presence of various phytoconstituents by qualitative analysis.

iv. To determine the total flavonoid content in various solvent extracts.

v. To determine the total phenol content in various solvent extracts.

vi. To determine the total tannin content in various solvent extracts.

vii. To evaluate the antioxidant and radical scavenging activity of various solvent extracts.

viii. To evaluate the chemopreventive and antioxidant activity of extract which showed a potent activity in invitro analysis against N-nitrosodiethylamine induced hepatocarcinogenesis in wistar rats by studying various parameters.

MATERIALS AND METHODS

Plant Material

The fresh leaves of *Cichorium intybus* Linn were collected from Vaniyambadi, Tamilnadu, India. The plant material was taxonomically indentified, confirmed and authenticated by Botanical Survery of India, Coimbatore, Tamilnadu and the voucher specimen (BSI/SRC/5/23/2012-13/Tech-1952) was retained in our laboratory for further reference. The collected leaves were shade dried and the dried materials were crushed to coarse powder with mechanical grinder. The powder was stored in an airtight container for extraction.

Animals

The animals used in this study were obtained from Agricultural University, Mannuthy, Thrissur, kerala (328/99/CPCSEA) and were housed in polypropylene cages. The animals were maintained under standard laboratory conditions ($25^{\circ} \pm 2^{\circ}C$; 12 hr light and dark cycle). The animals were fed with standard diet and water *ad libitum*. Ethical clearance (for handling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee (887/ac/05/CPCSEA) before performing the study on animals.

Extraction of *Cichorium intybus* leaves

The powdered leaves of *Cichorium intybus* were successively extracted in Soxhlet apparatus for 72 hours with solvents of increasing polarity i.e. petroleum ether (60-80° C), benzene, chloroform and methanol. After extraction, the solvent

was removed by distillation and evaporated under reduced pressure in a rotary evaporator to obtain crude extract. The dried extract thus obtained was stored in air tight glass container for further investigation.

Phytochemical screening

The extracts obtained were subjected to preliminary phytochemical screening (Kokate, 1994; Rosenthaler, 1930)

Phytochemical screening

Phytochemical examinations were carried out for all the extracts as per the standard methods.

Detection of alkaloids

Individually the extracts were dissolved in dilute Hydrochloric acid and filtered. The filtered extract was subjected to detection of alkaloids.

Mayer's Test

Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). The formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test

Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). The formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test

Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). The formation of red precipitate indicates the presence of alkaloids.

Hager's Test

Filtrates were treated with Hager's reagent (saturated picric acid solution). The formation of yellow coloured precipitate indicates alkaloids.

Detection of carbohydrates

The extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. The formation of the violet ring at the junction indicates the presence of Carbohydrates.

Benedict's Test

Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test

Filtrates were hydrolysed with dil HCl, neutralized with alkali and heated with Fehling's A & B solutions. The formation of red precipitate indicates the presence of reducing sugars.

Detection of glycosides

Individually the extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test

The extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test

The extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. The formation of pink to blood red colour indicates the presence of cardiac glycosides.

Keller Killiani Test

Small portion from the extract was shaken with 1ml of Glacial acetic acid containing trace of ferric chloride. 1ml of Conc H_2So_4 was added carefully by the sides of the test tube. A blue colour in the acetic acid layer and red color at the junction of two liquids indicate the presence of glycosides.

Detection of saponins

Froth Test

Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test

0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phytosterols

Libermann's Test

To 2ml filtrate in hot alcohol, few drops of acetic anhydride was added. Formation of brown precipitate indicates the presence of sterols.

Salkowski's Test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Libermann Burchard's test

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

Detection of phenols

Ferric Chloride Test

Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Detection of tannins

Lead acetate Test

To 2ml of filtrate, few drops of lead acetate solution were added in a test tube. Formation of yellow precipitate indicates the presence of Tannins.

Detection of flavonoids

Alkaline Reagent Test

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Detection of proteins and aminoacids

Millon's Test

2ml of filtrate was treated with 2ml of millon's reagent in a Test tube and heated in a water bath for 5 minutes, cooled and few drops of $NaNo_2$ was added. Formation of white precipitate, which turns to red upon heating, indicates the presence of proteins and amino acids.

Ninhydrin Test

To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

Biuret Test

2ml of filtrate was treated with 2ml of 10% sodium hydroxide in a test and heated for 10 minutes. A drop of 7% copper sulphate solution was added in the above mixture. Formation of purplish violent indicates the presence of Proteins.

Detection of Fixed oils and Fats

Oily Spot Test

One drop of extract was placed on filter paper and the solvent was evaporated. An oily stain of filter paper indicates the presence of fixed oil.

Quantitative estimation of bioactive compounds

Estimation of total phenolic content

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method. It is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). This method, based on the Slinkard and Singleton, 1977 and the early work of Singleton & Rossi, 1965 is a colorimetric oxidation/reduction method for phenolic compounds. A blue colour which is the product of metal oxidation, that exhibits a broad light absorption with a maximum at 764 nm. The intensity of light absorption is proportional to the concentration of phenols 20 μL of the diluted sample was added to 100 μL of Folin–Ciocalteu reagent. After 8 min, 300 μL of saturated sodium carbonate solution (25%) was added. The absorbance was measured at 764 nm. The calibration curve

was prepared with gallic acid solutions ranging from 10 to 1000 µg/ml, and the results are given as gallic acid equivalents (GAE).

Determination of total tannin content

The total tannin content was determined by modified method of Polshelttiwar et al., 2007. Different concentrations of extract (0.1 ml) was mixed with 0.5 ml of Folin-Denis reagent and followed by 1 ml of Na_2Co_3 (0.5% w/v) solution and distilled water (up to 5 ml). The absorbance was measured at 755 nm within 30 min of the reaction aginst the blank. The total tannin in the extract was expressed as the equivalent to tannic acid (g TAE/g extract).

Determination of total flavonoids

Flavonoid content was measured using aluminium chloride colorimetric method. Various concentrations of extract were mixed with 0.1 ml of 10 % aluminium chloride (w/v), 0.1 ml of 1 M potassium acetate and 2.8 ml distilled water. The mixture was allowed to stand at room temperature for 30 minutes. The absorbance of reaction mixture was measured at 415 nm. Results are expressed as mg/g quercetin equivalent (Chang, 2002).

Evaluation of total antioxidant capacity by phosphomolybdenum method

The antioxidant activities of various solvent extracts were evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al 1999. The assay is based on the reduction of Mo (VI) – Mo (V) by the extract. The subsequent formation of a green phosphate/Mo (V) complex at acid pH. 0.3 ml different concentrations of extract (10µg/ml to 200 µg/ml) were mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM

ammonium molybdate). 0.3 mL of methanol was used as blank in place of extracts. The tubes were incubated in a boiling water bath at 95°C for 90 min. The absorbance of the solution was measured at 695 nm after cooling to room temperature. The antioxidant capacity of each sample was expressed as ascorbic acid equivalent.

Determination of free radical scavenging activity

DPPH radical scavenging assay

Free radical scavenging activity was determined spectrophotometrically using the method of Blois, 1958. This method is based on the measurement of the reducing ability of antioxidants toward the DPPH radical. Briefly, 100 µl of various concentrations of the *Cichorium intybus* leaf extract (10 µg/ml to 400 µg/ml) in methanol were added to 10 ml of a methanol solution of DPPH ($1.01.10^{-2}$ M). The mixture was allowed to stand at room temperature for 30 min in the dark after a vigorous shake. The absorbance was measured at 517 nm. The control mixture consists of 100 µl of methanol and 10 ml of DPPH solution. The scavenging activity on the DPPH radical was calculated as inhibition percentage using the following equation:

$$\% \text{ Inhibition} = [(AB - AS)/AB] \times 100$$

where AB is the absorbance of the control reaction (containing all reagents except the test compound), and AS is the absorbance of the test compound. Ascorbic acid was used as reference standard. The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Nitric oxide scavenging activity

This method is based on principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which further interacts with oxygen to produce nitrite ion that can be estimated using Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitrite ion. For experimental, sodium nitroprusside (10 mM) in phosphate buffer saline (PBS pH 7.4) was mixed with different concentration of extract (10 μg/ml to 1000 μg/ml) dissolved in respective solvent and incubated at 25°C for 150 minutes. The same reaction mixture without extract but equivalent amount of solvent served as control. After incubation period 1.5 ml of the incubated solution was removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine was measured at 546 nm. Quercetin was used as positive control (Green et al., 1982). The tests were carried out in triplicate. The nitric oxide scavenging activity is calculated as

Scavenging activity = [{C-T/C} X 100]

where C = absorbance of control and T = absorbance of test solution.

Lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity of the *Cichorium intybus* leaf extracts were determined according to the method of Duh & Yen, 1997. Egg lecithin

(3 mg/ml phosphate buffer, pH 7.4) was sonicated in an ultrasonic sonicator for 10 min to ensure proper liposome formation. Test samples of *Smilax xeylanica* leaf extract (100 µl) of different concentrations (10 µg/ml to 1000 µg/ml) were added to liposome mixture (1 ml). Ferric chloride (10 µl, 400 mM) and L-ascorbic acid (10 µl, 200 mM) were added to induce lipid peroxidation. After 1 h incubation at 37° C the reaction was stopped by adding hydrochloric acid (2 ml, 0.25 N) containing trichloroacetic acid (150 mg/ml) and thiobarbutyric acid (3.75 mg/ml). The reaction mixture was boiled for 15 min, cooled, centrifuged at 1000 rpm for 15 min and the absorbance of the supernatant was measured at 532 nm. Tocopherol was used as reference standard. The control was without extract.

The scavenging effect was measured using the following equation

Scavenging effect (%) = [(A control - A sample) / A control] X 100.

Cupric ions (Cu^{2+}) reducing antioxidant capacity (CUPRAC assay)

By the method of Apak et al., 2004, the cupric reducing antioxidant capacity (CUPRAC) was determined. 1 ml of $CuCl_2$ solution ($1.0x10^{-2}$M), 1 ml ethanolic neocuproine solution ($7.5x10^{-3}$ M) and 1 ml NH_4CH_3COO (1M, pH 7.0) were added to a test tube and mixed. The *Cichorium intybus* leaf extract at different concentrations (10 µg/ml to 1000 µg/ml) was added to the initial mixture to make the final volume 4.1 ml and after 1 h, the absorbance was measured at 450 nm against a reagent blank. Increased reduction capability is denoted by increased absorbance of the reaction mixture.

Ferric reducing antioxidant capacity

According to the method described by Oyaizu, 1986 the reducing powers of extracts were determined. Different concentrations of *Cichorium intybus* leaf extracts (10 µg/ml to 1000 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). After incubation at 50°C for 20 min 2.5 ml of trichloroacetic acid (10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. Ascorbic acid was used as reference standard.

Metal chelating activity

According to the method of Dinis *et al.,* 1994, the chelation of ferrous ions by extracts was estimated. 50 µl of 2 mM Ferric chloride was added to 1 ml of different concentrations of the *Cichorium intybus* leaf extract (10 µg/ml to 1000 µg/ml). By the addition of 0.2 ml of 5 mM ferrozine solution the reaction was initiated. The mixture was allowed to stand at room temperature for 10 min after vigorous shaking. The absorbance of the solution was measured at 562 nm. The percentage inhibition of ferrozine-Fe2+ complex formation was calculated as [(A control- A- sample)/ A- sample] ´X 100, where A- control denotes absorbance of the control, and A- sample denotes absorbance of the extract/ standard. Na_2EDTA was used as positive control.

Acute oral toxicity study of chloroform extract of *cichorium intybus* leaf extract

Animals:

Swiss albino mice of female sex weighing 20-25 gms were used for the study. The animals were housed in polypropylene cages. The animals were maintained under standard laboratory conditions (25º ± 2ºC; 12hr light and dark cycle). The animals were fed with standard diet and water *ad libitum*. Ethical clearance (for handling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee before performing the study on animals.

Acute Toxicity Test:

Acute oral toxicity study for chloroform extract of *cichorium intybus* was carried out as per OECD guideline 425 (Up and Down procedure). The test procedure minimizes the number of animals required to estimate the acute oral toxicity. The test allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Animals were fasted (food but not water was with held overnight) prior to dosing. The fasted body weight of each animal was determined and the dose was calculated according to the body weight.

Limit Test at 2000 mg/kg

The drug was administered in the dose of 2000 mg/kg body weight orally to one animal. If the test animal survived, then four animals were dosed sequentially; therefore, a total of five animals were tested. Animals were observed individually at least once during the first 30 minutes after dosing, periodically during the first 24 hours (with special attention given during the first 4 hour), and daily thereafter, for a total of 14 days. After the experimental period, the animals

were weighed and humanely killed and their vital organs including heart, lungs, liver, kidneys, spleen, adrenals, sex organs and brain were grossly examined (OECD, 425)

Evaluation of chemopreventive and antioxidant activity of chloroform extract of *Cichorium intybus* leaf extract against N-nitrosodiethylamine induced hepatocarcinogenesis

Experimental design and induction of hepatocellular carcinoma (HCC)

Healthy male albino wistar rats (6-8 weeks) weighing 130-150 gms were used for the study. Randomization in selection of animals for grouping was carried out to avoid statistical difference in the body weight of animals. The experimental animals were divided into six groups, each group comprising of six animals. The experimental animals were divided into six groups of six animals each according to the following experimental regimen. Group 1 animals served as normal control rats. Group 2 animals received *Cichorium intybus* leaf extract 200 mg/kg/day in 0.5% carboxy methyl cellulose (CMC) orally for 16 weeks. Group 3 animals received *Cichorium intybus* leaf extract 400mg/kg/day in 0.5% CMC orally for 16 weeks. Group 4 animals received vehicle 0.5% CMC 2ml/kg/day orally started 1 week prior to single intraperitoneal injection of NDEA 200mg/kg and after two weeks recovery carcinogenic effect was promoted by Phenobarbital (0.05%, PB). Promoter was supplemented to the animals through drinking water up to 14 successive weeks. The oral administration of vehicle was continued after NDEA injection throughout the experimental period of 16 weeks. Group 5 animals were given *Cichorium intybus* leaf extract 200mg/kg/day in 0.5% CMC orally started1 week prior to the injection of NDEA and 2 weeks later the promoter phenobarbitone was incorporated into the

drinking water at the concentration of 0.05% for 14 successive weeks. The oral administration of extract was continued for 16 weeks after NDEA injection. Animals in group 6 were given *Cichorium intybus* leaf extract 400mg/kg/day in 0.5% CMC orally started 1 week prior to the injection of DENA and 2 weeks later the promoter phenobarbitone was incorporated into the drinking water at the concentration of 0.05% for 14 successive weeks. The oral administration of extract was continued for 16 weeks after NDEA injection. Body weight was recorded at the end of every week for 16 weeks (Singh *et al.,* 2004).

Sample collection

At the end of the experimental period (16 weeks), blood samples were collected from retro-orbital plexus under anaesthesia in EDTA tubes and centrifuged at 2200×g for 15 min. at 4°C. Plasma samples were stored at -20°C for biochemical analysis.

Biochemical analysis

By the method described by Reitman and Frankel, 1957 serum transaminases (AST and ALT) were determined. By the method of Kind and King, 1954 Serum alkaline phosphatase (ALP) was estimated. By the method described by Malloy and Evelyn, 1937 and wooten, 1964 serum bilirubin (SB) and total protein (TB) were estimated. According to the method described by Szas, 1976, gamma glutamyl transferase (GGT) activity was determined. By using the enzyme immunoassay kit, α-feto protein (AFP) and carcinoembryonic antigen (CEA), the tumor markers were estimated based on solid phase enzyme linked immunosorbent assay (ELISA) (Sell, 1978; Macnab, 1978).

After blood collection animals were sacrificed by cervical decapitation and the liver was excised, washed in ice cold saline, blotted to dryness and examined for any deductable changes. A portion of liver tissue was then homogenized for biochemical assays.

Preparation of tissue homogenate

The liver tissue was weighed and by using 0.025 M Tris–HCl buffer, 10% tissue homogenate was prepared at pH 7.5. The clear supernatant after centrifugation at 10,000 rpm was used to measure TBARS. By the method described by Folch *et al.,*1957, the vitamin E level was determined. The tissues were weighed and using chloroform–methanol mixture ($CHCl_3$:CH_3OH) (2:1; v/v) lipids were extracted from tissues. This extract was used for the estimation of vitamin E. The tissues were minced and homogenized (10% w/v) in 0.1 M phosphate buffer (pH 7.0) and centrifuged for 10 min and the resulting supernatant was used for the estimation of non-enzymatic and enzymatic antioxidants.

Estimation of Lipid peroxidation (LPO)

By the method described by Ohkawa et al., 1979, the levels of thiobarbituric acid reactive substances in the liver was measured. The portion of liver homogenates was mixed with 0.2 ml of 8.1% Sodium dodecyl sulphate, 1.5 ml of 20% acetic acid and 1.5 mL of 0.8% thiobarbituric acid, then the volume was adjusted to 4.0 ml with distilled water. With 1.0 ml of distilled water and 5.0 ml of n- butanol and pyridine (15:1 v/v) the reaction mixture was extracted and the absorbance was measured in organic layer at 532 nm after centrifugation.

Estimation of superoxide dismutase (SOD) activity

After diluting the 0.5 ml of tissue homogenate with water, 2.5 ml of ethanol and 1.5 ml of chloroform were added and mixed for 1 min at 4 °C and centrifuged. The supernatant was separated and determined for enzyme activity. Appropriately diluted enzyme preparation is mixed with 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of 186 µM PMS, 0.3ml of 30 µM Nitroblue tetrazolium (NBT), 0.2 ml of 780 µM NADH, and water in a total volume of 3 ml. By addition addition of NADH, the reaction was started. After the reaction, by the addition of 1 ml glacial acetic acid and incubation at 30°C for 90 seconds, the reaction was stopped. With 4 ml of n-butanol, the reaction mixture was stirred vigorously and shaken. At 560 nm, the intensity of the chromogen was measured in the butanol layer against butanol blank. A system devoid of enzyme served as control (Kakkar et al., 1984).

Estimation of catalase activity

0.1 ml of tissue homogenate is mixed with 1.0 ml of 0.01M phosphate buffer (pH 7.0), and 0.4 ml of 2M H_2O_2. By the addition of 2.0 ml of dichromate-acetic acid reagent the reaction was stopped (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). The absorbance was measured at 620 nm. Catalase activity was expressed as nm of H_2O_2 consumed/min/mg protein (Sinha, 1972).

Reduced glutathione (GSH)

By the method of Ellman, 1959, GSH was estimated. By using 2 ml of 5% Trichloro acetic acid, 0.5 ml of tissue homogenate was precipitated. 1 ml of

supernatant was taken after centrifugation and mixed with 0.5 mL of Ellman's reagent and 3 ml of phosphate buffer. The absorbance of colour developed was measured at 412 nm.

Ellman's reagent- 19.8 mg of 5,5' dithio (bis) nitrobenzoic acid in 100 ml of 1% sodium citrate).

Histopathological examination

A portion of liver tissue was subjected to histopathological examination. A portion is fixed in 10% formalin and embedded in molten paraffin wax and were ultra sectioned (5-6 μm thickness). The tissues were stained with hematoxylin and eosin (H & E) and were examined under light microscope for histopathological changes.

Statistical analysis

Results were expressed as mean ± standard error of mean (SEM). The results were analysed for statistical significance by one way ANOVA followed by dunnett's test (Graphpad Software Inc,La Jolla, CA. Trial version). The criterion for statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Table 1. Percentage extraction yield of powdered leaves of *Cichorium intybus* in different organic solvents

Solvent	Percentage yield (%w/w)
Petroleum ether	3.68 %
Chloroform	3.20 %
Ethyl acetate	3.33 %
Ethanol	13.18 %

Table 2. Qualitative phytochemical screening of various solvent extracts of *Cichorium intybus* leaves

Phytochemicals	Test	Pet ether extract	Chloroform extract	Ethyl acetate extract	Ethylalcohol extract
Alkaloids	a. Mayer's test	+	+	+	+
	b. Dragendroff's	+	+	+	+
	c. Hager's test	+	+	+	+
	d. Wagner's test	+	+	+	+
Phytosterols and Triterpenoids	a. Leibermann's test	–	+	+	+
	b. Leiberman-Burchard	–	+	+	+
	c. Salkowaski test	–	+	+	+
Flavonoids	a. Alkaline reagent test	–	+	+	+
Saponins	a. Foam test	–	–	–	+
Proteins and Aminoacids	a. Millon's test	–	–	+	+
	b. Ninhydrin test	–	–	+	+
	c. Biuret test	–	–	+	+
Phenolics and Tannins	a. Ferric chloride test	–	+	+	+
	b. Lead acetate test	–	+	+	+
Carbohydrate	a. Molisch's test	–	–	+	+
	b. Fehling's test	–	–	+	+
	c. Benedict's test	–	–	+	+
Gylcosides	a. Modified Borntrager's test	–	–	+	+
	b. Legal's test	–	–	–	–
	c. Keller-Killiani test	–	–	+	+

(+) Present, (-) Absent

Table 3. Total Phenolic content (TPC), Total Tannin content (TTC), Total Flavonoid content (TFC) and Total antioxidant capacity (TAC) of the different extracts of *Cichorium intybus* leaves

Extract	TPC (µg of GAE/mg of extract)	TTC (µg of TAE/mg of extract)	TFC (µg of quercetin/mg of extract)	TAC (µg of ascorbic acid/mg of extract)
Petroleum ether	17.07	15.73	124.22	633.30
Chloroform	51.67	80.88	428.97	1450.59
Ethyl acetate	34.60	48.74	28.01	104.47
Ethanol	42.90	64.38	22.67	56.40

Each sample is analysed thrice (n=3)

Statistical analysis by one-way ANOVA followed by Tukey multiple comparison tests.

Values significantly differ ($P<0.0001$) from the other extract in same row.

Total Phenolic content (TPC) is expressed as milligram of gallic acid equivalent per gram of sample. Total tannin content (TTC) is expressed as milligrams of tannic acid equivalents per gram of dry extract. Total Flavonoid content (TFC) is expressed as milligrams of quercetin equivalents per gram of dry extract. Total antioxidant capacity is expressed as milligrams of ascorbic acid equivalents per gram of dry extract.

Table 4. Free radical scavenging activity of different extracts from leaves of *Cichorium intybus* (IC_{50} (µg/mL expressed as mean ± S.D)

Extract	DPPH radical scavenging (µg/mL)	Nitric oxide scavenging (µg/mL)	Lipid peroxidation Inhibitory activity (µg/mL)
Petroleum ether	> 1000	5.26	19.55
Chloroform	> 1000	4.30	12.37
Ethyl acetate	169.64	3.71	9.83
Ethanol	204	81.30	32.57
Standard (Vitamin E)	6.80	7.08	2.03

IC_{50} values were expressed as µg/mL concentration (n=3). Lower IC_{50} values indicate higher radical scavenging activity. Values are significantly different from standard; ns-non significant; $^{*}P<0.05$;$^{**}P<0.01$;$^{***}P<0.001$. (ANOVA, followed by Dunnett's test)**.** Ascorbic acid was used as a reference for DPPH radical scavenging activity. Quercetin was used as a reference for nitric oxide scavenging activity. Tocopherol was used as a reference for Lipid peroxidation inhibitory activity. Na_2EDTA was used as a reference for metal chelating activity inhibitory activity.

Table 5. Cupric ions (Cu^{2+}) reducing capacity (CUPRAC assay) of various solvent extracts of *Cichorium intybus* leaves.

Extract	CUPRAC µg of Trolox/mg of extract
Petroleum ether	**5866.52**
Chloroform	**13398.87**
Ethyl acetate	**991.617**
Ethanol	**549.439**

Figure. 6 DPPH scavenging activity

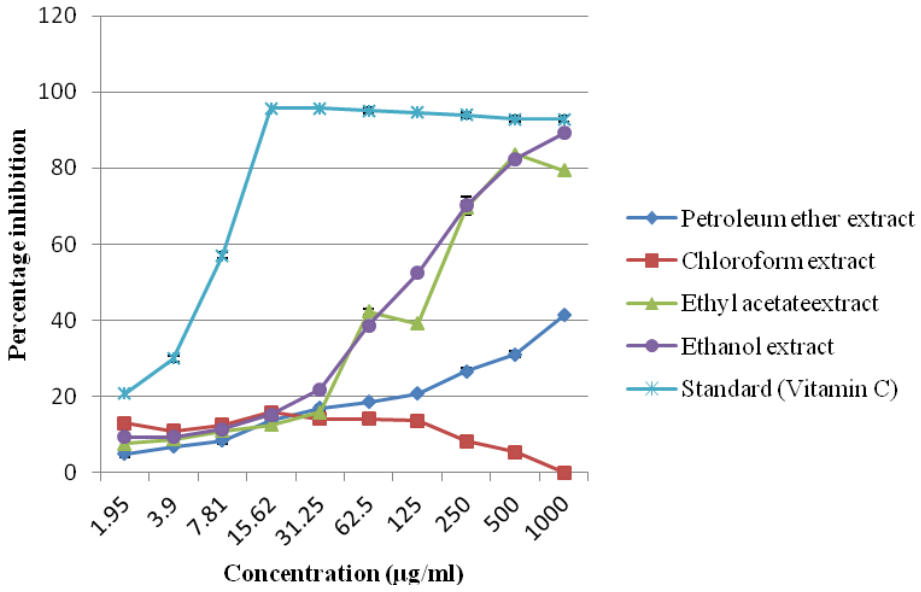


Figure. 7 Nitric oxide scavenging activity

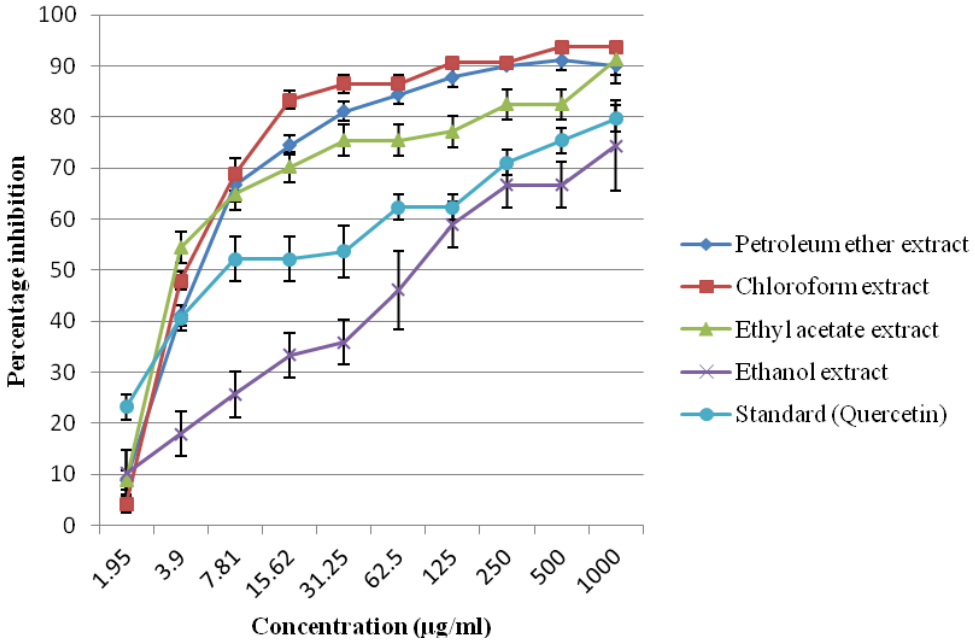


Figure. 8 Lipid peroxidation inhibitory activity

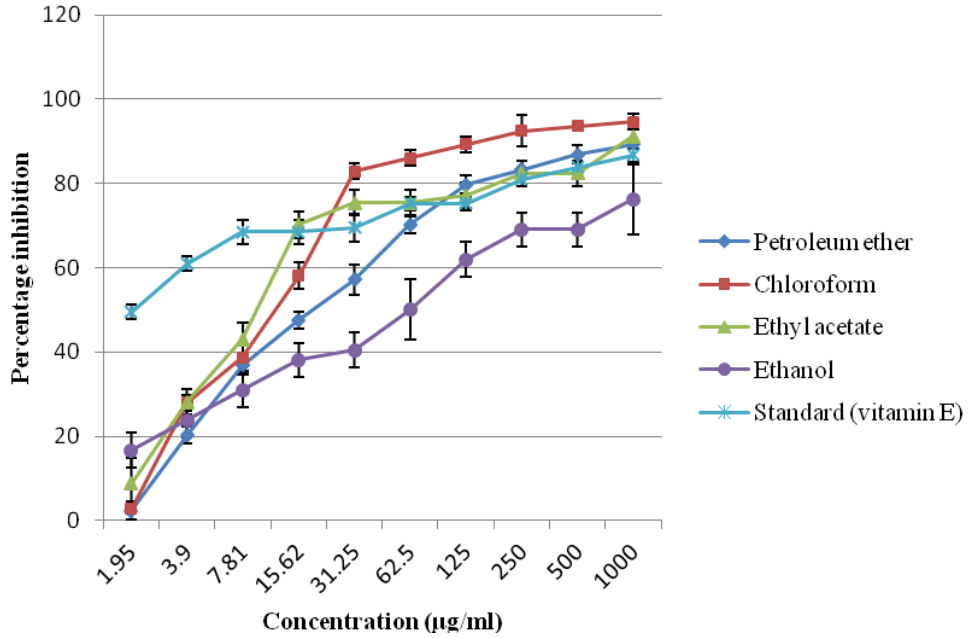


Figure. 9 Ferric reducing antioxidant capacity

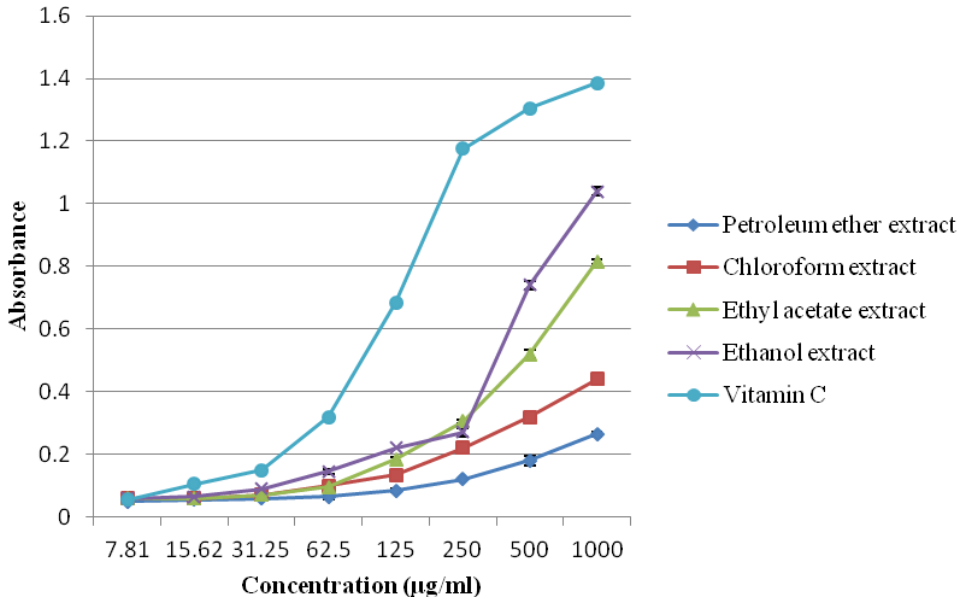


Figure. 10 Metal chelating activity

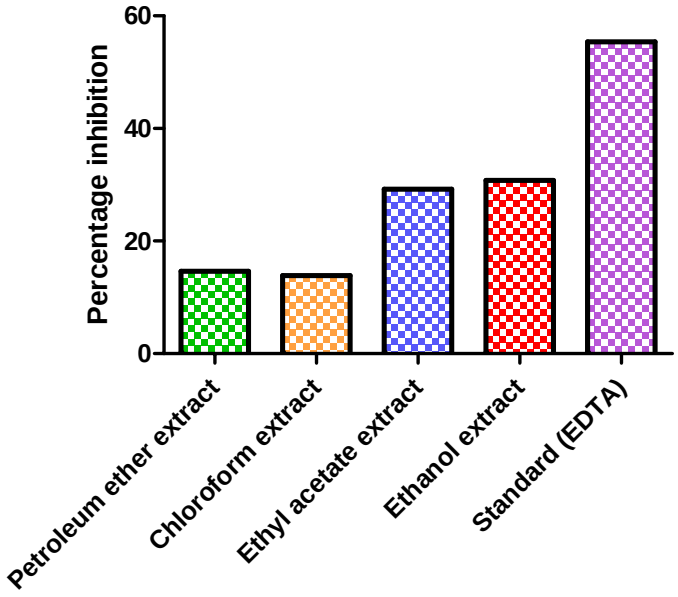


Table.6 Acute oral Toxicity study (425) observations.

Respiratory blockage in nostril	
Dyspnoea	Nil
Apnoea	Nil
Tachypnea	Nil
Nostril discharge	Nil
Motor activities	
Locomotion	Normal
Somnolence	Nil
Loss of righting reflex	Nil
Anaesthesia	Nil
Catalepsy	Nil
Ataxia	Nil
Toe walking	Nil
Prostration	Nil
Fasciculation	Nil
Tremor	Nil
Convulsion	
Clonic/tonic/tonic-clonic convulsion	Nil
Asphyxial convulsion	Nil
Opistotones (titanic spasm)	Nil

Reflexes	
Corneal	Normal
Eyelid closure	Normal
Righting	Normal
Light	Normal
Auditory and sensory	Normal
Ocular signs	
Lacrimation	Nil
Miosis	Nil
Mydriasis	Nil
Ptosis	Nil
Chromodacryorrhea	Nil
Iritis	Nil
Conjunctivitis	Nil
Salivation	
Saliva secretion	Nil
Piloerection	
Contraction of erectile tissue	Nil
Analgesia	
Decrease in reaction to induced pain	Nil
Muscle tone	
Hypo or hypertonia	Nil
Git sign	
Solid dried / watery stool	Nil
Emesis	Nil
Red urine	Nil
Skin	
Oedema	Nil

Figure. 11

Effect of *Cichorium intybus* leaf extract on body weight gain during NDEA induced hepatocarcinogenesis in rats

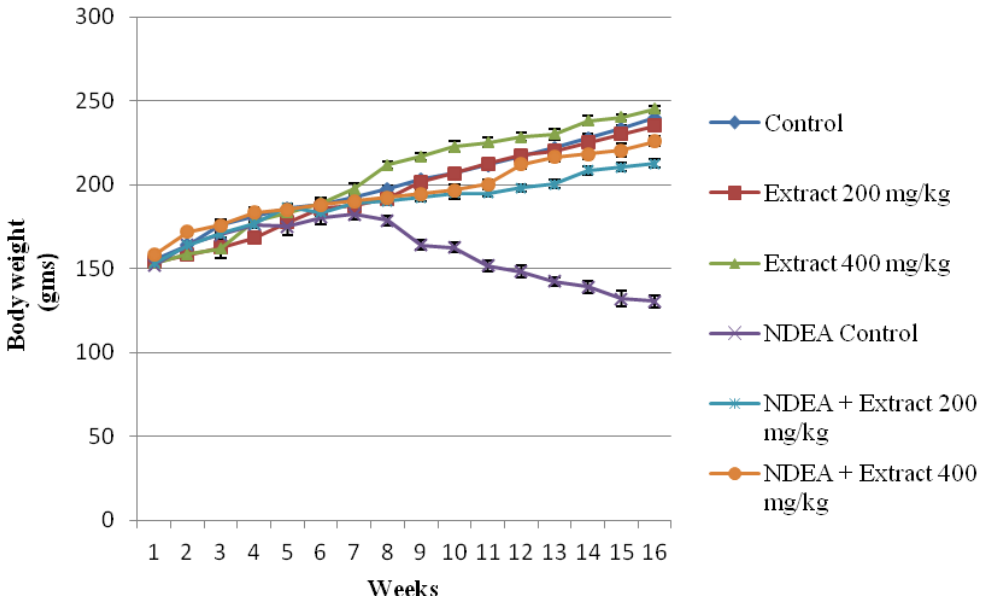
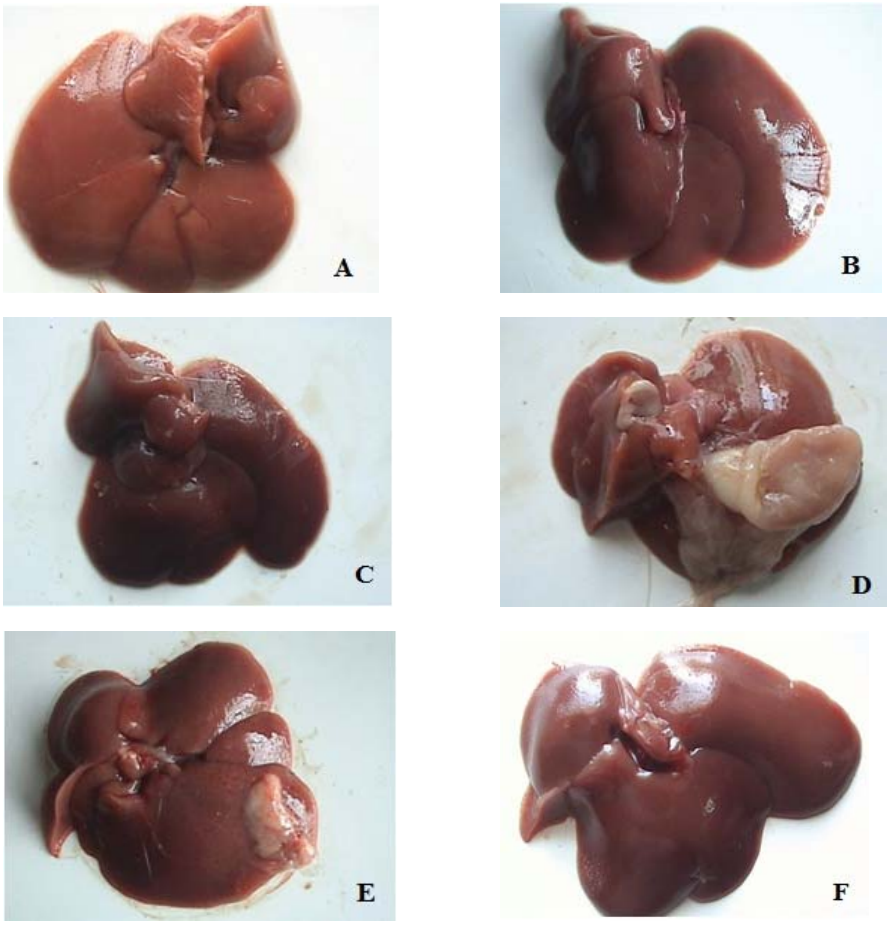


Fig. 12 Gross morphology of liver



A - Liver of control animal showing normal morphology. B and C - Liver of animal dosed with extract alone 200 mg/kg and 400 mg/kg respectively showing normal appearance which is indicates nontoxic nature of extract. D - Liver of group IV NDEA induced untreated animals (NDEA control) showing multiple lesions (whitish) with almost the entire surface of liver is occupied with abnormal growth. E- Liver of NDEA induced group V animals treated with *Cichorium Intybus* leaf extract 200 mg/kg showing marked reduction in damage caused by NDEA. F- Liver of rats treated with *Cichorium Intybus* leaf extract 400 mg/kg showing normal

morphology similar to control animals. No detectable lesions or nodules were noted in anterior and posterior surface of treatment groups.

Table. 7 Relative liver weights of various groups of rats at the end of the study (16 weeks)

GROUP	Relative liver weight (g liver/100 g body wt)
Group I (Control) (0.5%CMC) 2 ml/kg p.o.	2.22 ± 0.01
Group II (Extract 200mg/kg p.o.)	$2.18 \pm 0.03^{a\ ns}$
Group III (Extract 400mg/kg p.o.)	$2.23 \pm 0.03^{a\ ns}$
Group IV (NDEA alone)	$2.95 \pm 0.06^{a\ ***}$
Group IV (NDEA + Extract 200 mg/kg p.o.)	$2.59 \pm 0.05^{b\ ***}$
Group IV (NDEA + Extract 400 mg/kg p.o.)	$2.52 \pm 0.04^{b\ ***}$

All values are expressed as mean ± S.E.M, n=6 in each group.

[a] Values are significantly different from control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

[b] Values are significantly different from NDEA- induced group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001 **(**one-way ANOVA followed by Dunnett's test).

Figure. 13 Relative liver weights of various groups of rats at the end of the study (16weeks)

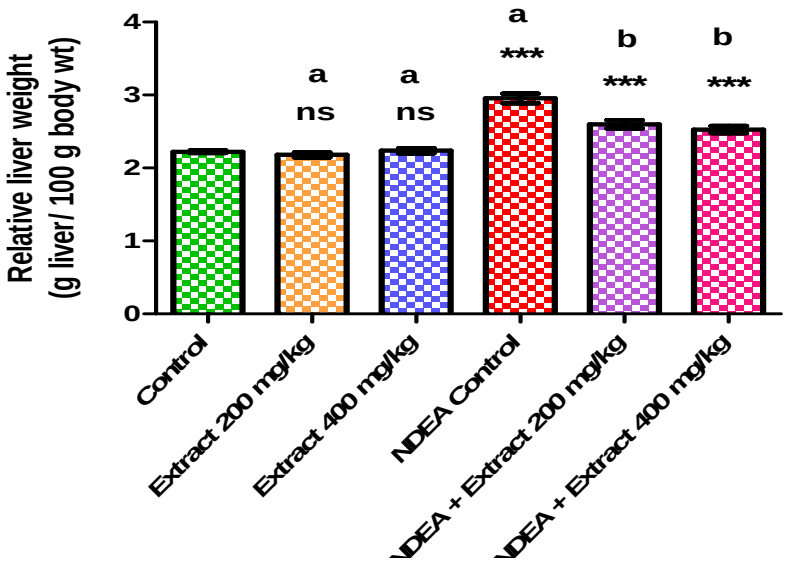

Relative liver weight
(g liver/ 100 g body wt)
4
3
2
1
0
a
ns
a
ns
a
***
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

Table.8 Effect of *Cichorium intybus* leaf extract on the activities of marker enzymes in the serum of control and experimental groups of rats

GROUP	AST (SGOT) (IU/L)	ALT (SGPT) (IU/L)	ALP (IU/L)	TB (mg/dl)	TP (mg/dl)	GGT (IU/L)
Group I (Control) (0.5%CMC) 2 ml/kg p.o.	11.47 ± 0.17	110.0 ± 0.92	215.3 ± 3.60	0.52 ± 0.01	7.88 ± 0.21	12.67 ± 0.20
Group II (Extract 200mg/kg p.o.)	^{a} 12.13 ± 0.20 ^{ns}	^{a} 111.20 ± 0.85 ^{ns}	^{a} 207.5 ± 3.86 ^{ns}	^{a} 0.51 ± 0.01 ^{ns}	^{a} 8.03 ± 0.20 ^{ns}	^{a} 12.12 ± 0.23 ^{ns}
Group III (Extract 400mg/kg p.o.)	^{a} 11.90 ± 0.37 ^{ns}	^{a} 108.20 ± 2.04 ^{ns}	^{a} 217.9 ± 3.90 ^{ns}	^{a} 0.52 ± 0.01 ^{ns}	^{a} 7.41 ± 0.17 ^{ns}	^{a} 12.20 ± 0.33 ^{ns}
Group IV (NDEA alone)	^{a} 69.33 ± 0.20 ***	^{a} 436.40 ± 10.38 ***	^{a} 465.80 ± 9.93 ***	^{a} 1.76 ± 0.09 ***	^{a} 4.78 ± 0.21 ***	^{a} 56.38 ± 2.35 ***
Group IV (NDEA + Extract 200 mg/kg p.o.)	^{b} 16.15 ± 0.20 ***	^{b} 162.60 ± 3.15 ***	^{b} 232.50 ± 6.40 ***	^{b} 0.70 ± 0.04 ***	^{b} 5.48 ± 0.17 ***	^{b} 23.68 ± 0.90 ***
Group IV (NDEA + Extract 400 mg/kg p.o.)	^{b} 12.17 ± 0.46 ***	^{b} 121.80 ± 1.63 ***	^{b} 221.5 ± 4.80 ***	^{b} 0.59 ± 0.03 ***	^{b} 6.33 ± 0.16 ***	^{b} 15.22 ± 0.44 ***

All values are expressed as mean ± S.E.M, n=6 in each group. One -way ANOVA followed by Dunnett's test was used to compare experimental groups.

[a] Values are significantly different from control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

[b] Values are significantly different from NDEA- induced group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Table. 9 Effect of *Cichorium intybus* leaf extract on α-feto protein levels (AFP) and carcino embryonic antigen (CEA) levels in the serum of control and experimental groups of rats.

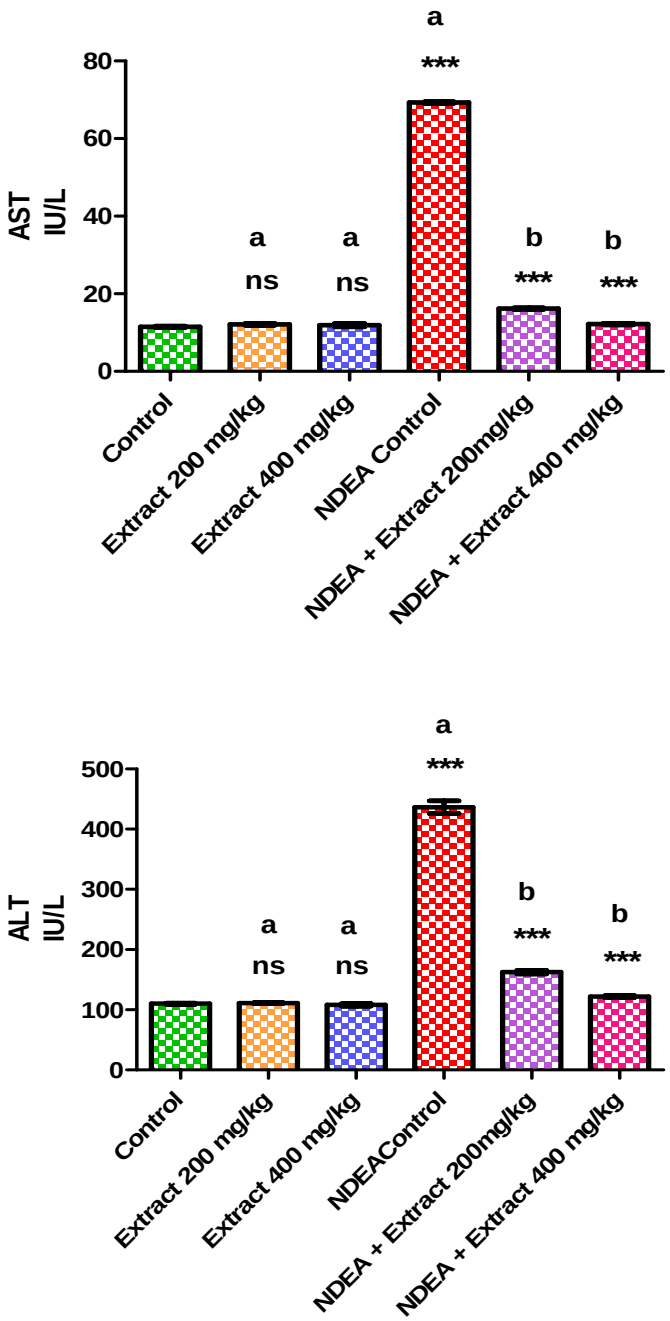
GROUP	α-feto protein (AFP) ng/ml	Carcino embryonic Antigen (CEA) ng/ml
Group I (Control) (0.5%CMC) 2 ml/kg p.o.	0.52 ± 0.03	1.5 ± 0.14
Group II (Extract 200mg/kg p.o.)	$0.51 \pm 0.02^{a\ ns}$	$1.46 \pm 0.15^{a\ ns}$
Group III (Extract 400mg/kg p.o.)	$0.49 \pm 0.02^{a\ ns}$	$1.62 \pm 0.15^{a\ ns}$
Group IV (NDEA alone)	$0.89 \pm 0.03^{a\ ***}$	$5.85 \pm 0.14^{a\ ***}$
Group IV (NDEA + Extract 200 mg/kg p.o.)	$0.71 \pm 0.02^{b\ ***}$	$4.08 \pm 0.24^{b\ ***}$
Group IV (NDEA + Extract 400 mg/kg p.o.)	$0.59 \pm 0.03^{b\ ***}$	$2.44 \pm 0.14^{b\ ***}$

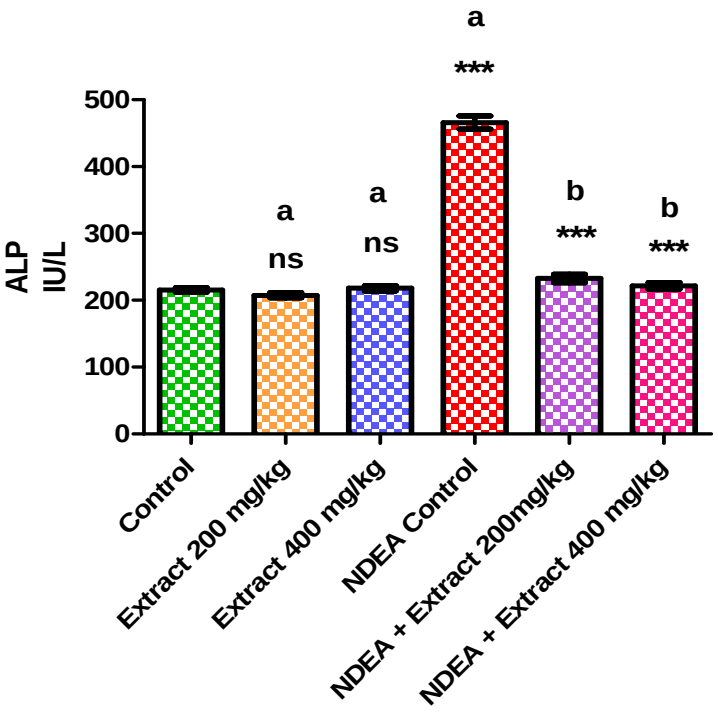
All values are expressed as mean ± S.E.M, n=6 in each group. One -way ANOVA followed by Dunnett's test was used to compare experimental groups. [a] Values are significantly different from control group; n

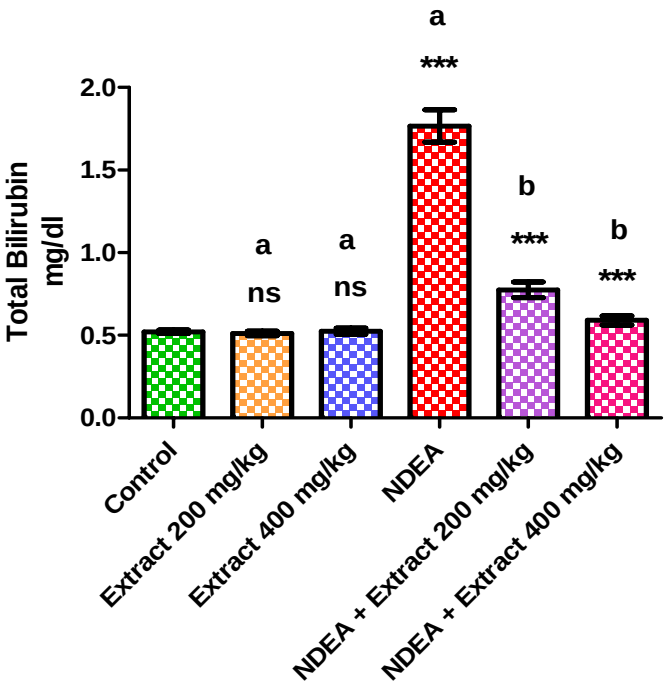
s-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

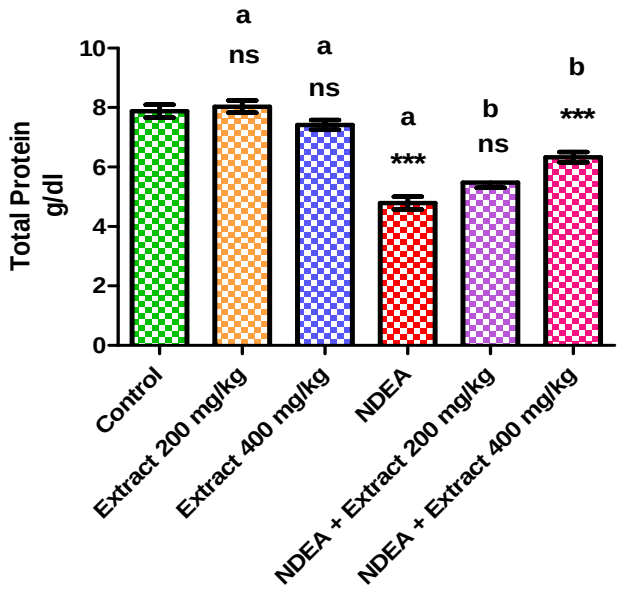
[b] Values are significantly different from NDEA- induced group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

Figure. 14 Effect of *Cichorium intybus* leaf extract on the activities of marker enzymes in the serum of control and experimental groups of rats




ALP
IU/L
500
400
300
200
100
0
a
***
a
ns
a
ns
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200mg/kg
NDEA + Extract 400 mg/kg


Total Bilirubin
mg/dl
2.0
1.5
1.0
0.5
0.0
a
***
a
ns
a
ns
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg


Total Protein
g/dl
10
8
6
4
2
0
a
ns
a
ns
a
***
b
ns
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

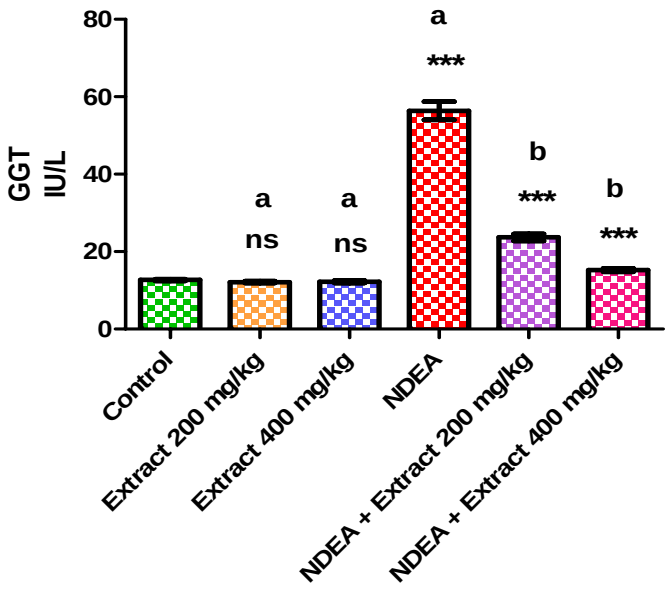
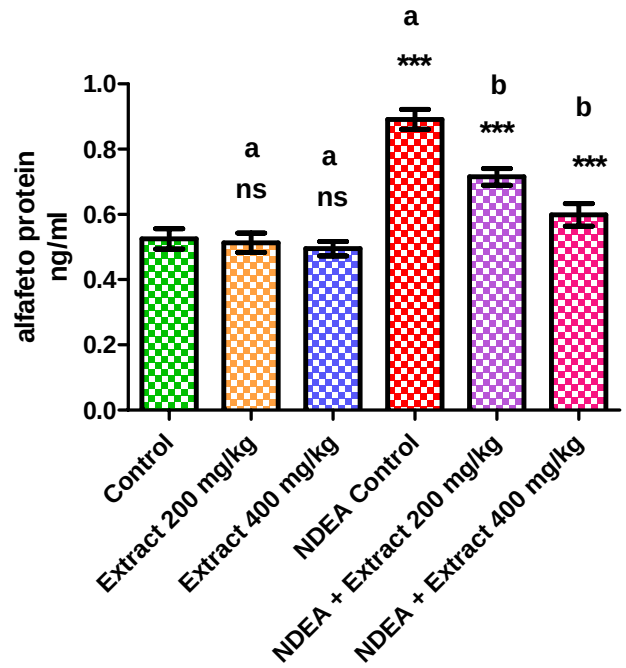

GGT
IU/L
80
60
40
20
0
a
ns
a
ns
a
***
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

Figure. 15 Effect of *Cichorium intybus* leaf extract on α-feto protein levels (AFP) and carcino embryonic antigen (CEA) levels in the serum of control and experimental groups of rats.


alfafeto protein
ng/ml
1.0
0.8
0.6
0.4
0.2
0.0
a
ns
a
ns
a
***
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

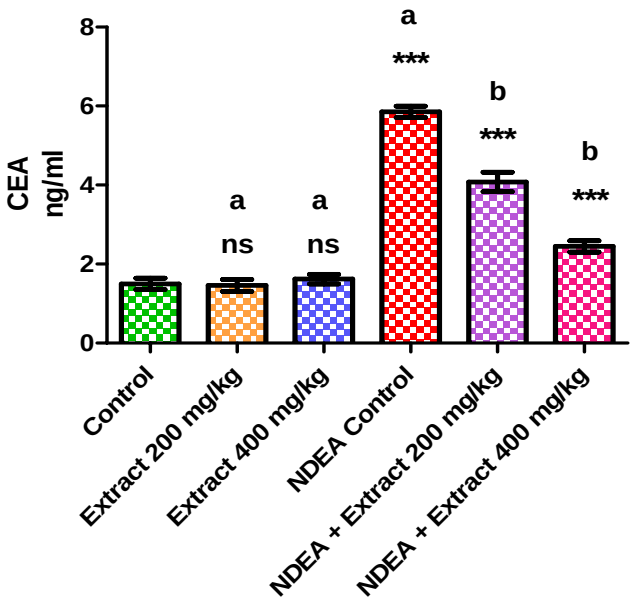

CEA
ng/ml
8
6
4
2
0
a
ns
a
ns
a
***
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

Table. 10 Effect of *Cichorium intybus* leaf extract on lipid peroxidation and the status of enzymatic and non-enzymatic antioxidants in liver of control and experimental group of animals

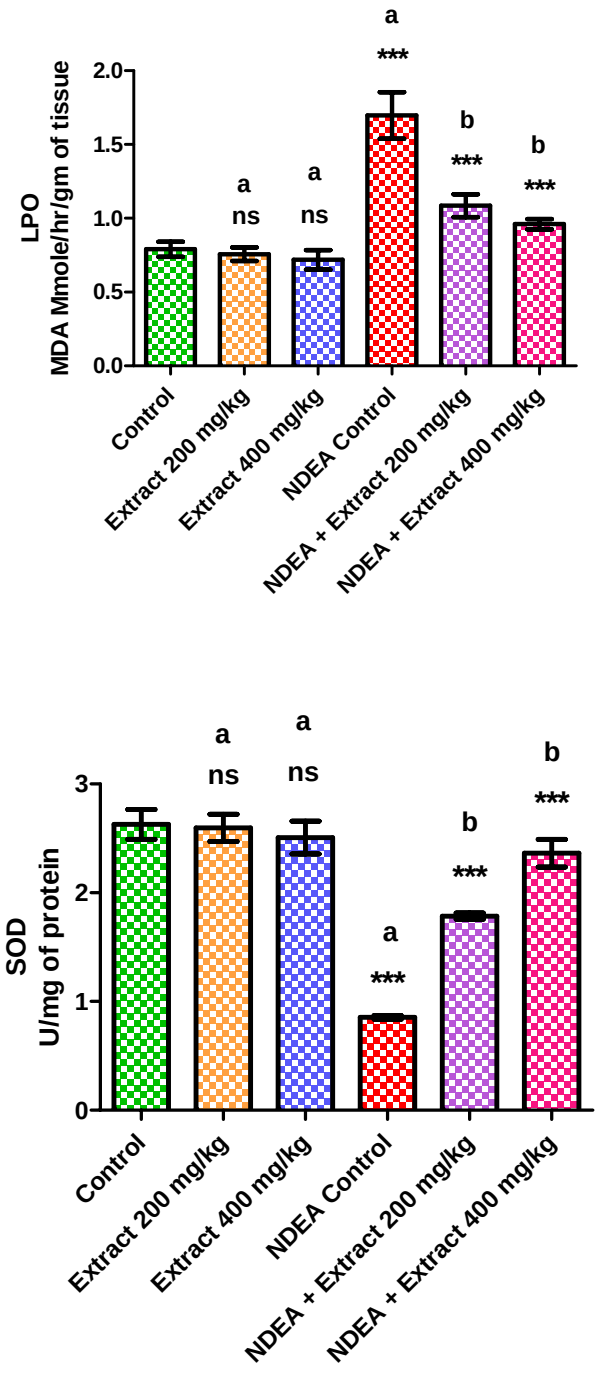
Parameter	Group I (Control)	Group II Extract (200mg/kg)	Group III Extract (400mg/kg)	Group IV B(a)P	Group V B(a)P + Extract (200mg/kg)	Group VI B(a)P + Extract (400 mg/kg)
LPO	0.79 ± 0.05	$0.75 \pm 0.04^{a\ ns}$	$0.71 \pm 0.06^{a\ ns}$	$1.69 \pm 0.15^{a\ ***}$	$1.08 \pm 0.07^{a\ ***}$	$0.96 \pm 0.03^{a\ ***}$
SOD	2.62 ± 0.13	$2.59 \pm 0.12^{a\ ns}$	$2.50 \pm 0.15^{a\ ns}$	$0.85 \pm 0.01^{a\ ***}$	$1.78 \pm 0.03^{b\ ***}$	$2.36 \pm 0.12^{b\ ***}$
Catalase	1.94 ± 0.11	$2.01 \pm 0.11^{a\ ns}$	$2.02 \pm 0.11^{a\ ns}$	$0.85 \pm 0.11^{a\ ***}$	$1.14 \pm 0.08^{b\ *}$	$1.41 \pm 0.09^{b\ ***}$
GSH	2.26 ± 0.07	$2.23 \pm 0.08^{a\ ns}$	$2.34 \pm 0.02^{a\ ns}$	$0.91 \pm 0.02^{a\ ***}$	$1.74 \pm 0.04^{b\ ***}$	$1.93 \pm 0.04^{b\ ***}$

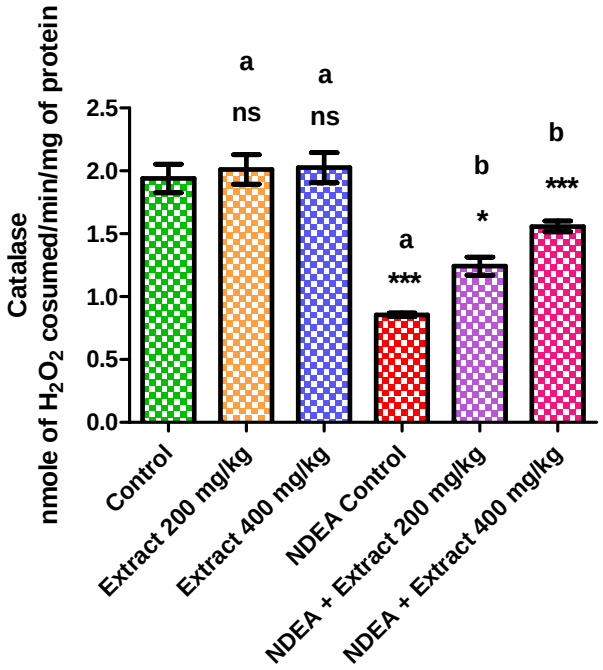
All values are expressed as mean ± S.E.M, n=6 in each group

[a] values are significantly different from control group; ns-non significant; *P < 0.05; **P < 0.01; ***P < 0.001.

[b]values are significantly different from NDEA control group; ns-non significant; *P<0.05;**P<0.01;***P<0.001. (ANOVA, followed by Dunnett's test)

Figure. 16 Effect of *Cichorium intybus* leaf extract on lipid peroxidation and the status of enzymatic and non-enzymatic antioxidants in liver of control and experimental group of animals




Catalase
nmole of H_2O_2 cosumed/min/mg of protein
2.5
2.0
1.5
1.0
0.5
0.0
a
ns
a
ns
a
***
b
*
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

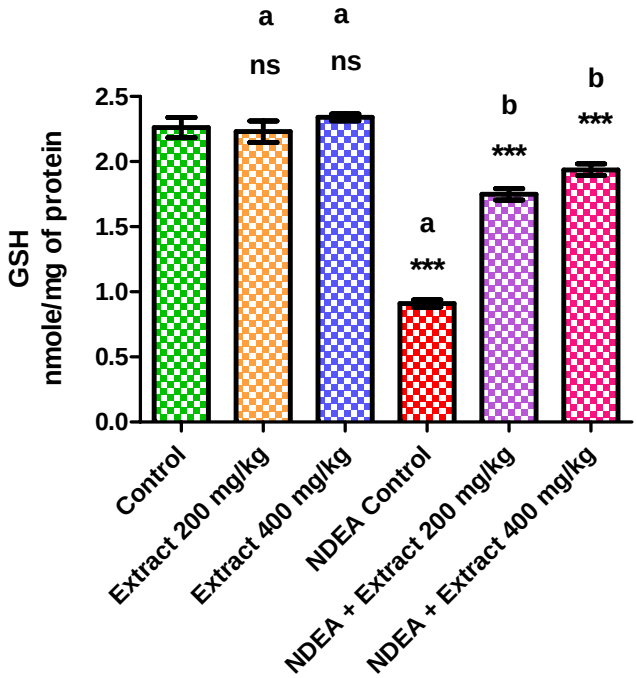
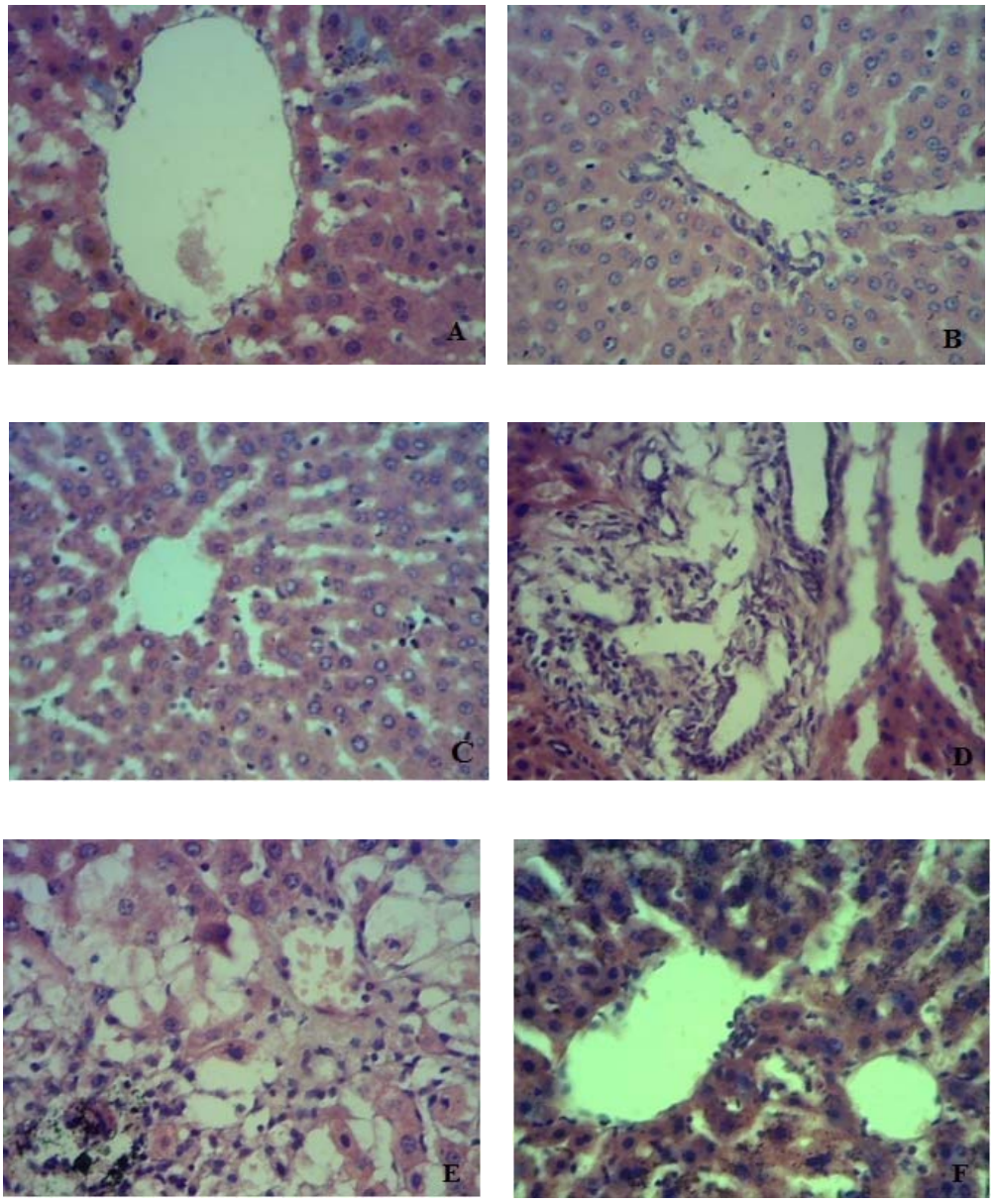

GSH
nmole/mg of protein
2.5
2.0
1.5
1.0
0.5
0.0
a
ns
a
ns
a
***
b
***
b
***
Control
Extract 200 mg/kg
Extract 400 mg/kg
NDEA Control
NDEA + Extract 200 mg/kg
NDEA + Extract 400 mg/kg

Figure. 17 Histopathological image of liver tissues



Percentage yield

The percentage yield of extract obtained from extraction of powdered leaf material of *Cichorium intybus* using solvents of increasing polarity was found to be 3.68 % w/w using petroleum ether, 3.20 % w/w using chloroform, 3.33 % using ethyl acetate and 13.18 % using ethanol as solvent (Table 1).

Phytochemical study

The phytochemical examination of petroleum ether extract revealed the presence of alkaloids, chloroform extract revealed the presence of alkaloids, flavonoids, phenolics and tannins, phytosterols and triterpinoids. Ethyl acetate extract revealed the presence of alkaloids, phytosterols and triterpinoids, flavonoids, proteins and aminoacids, phenolics and tannins, carbohydrates and glycosides and ethanol extract revealed the presence of alkaloids, phytosterols and triterpinoids, flavonoids, proteins and aminoacids, phenolics and tannins, carbohydrates, glycosides and saponins (Table 2).

Quantitative estimation of bioactive compounds

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu method and the results were expressed as gallic acid equivalents (GAE). The total phenolic content in chloroform extract was found to be high which showed a content of 51.67 mg GAE/gm of dry extract followed by ethanol extract which showed 42.90 mg GAE/gm of dry extract. Ethyl acetate extract showed a total phenolic content of 34.60 mg GAE/gm of dry extract and petroleum extract showed a least phenolic content of 17.07 mg GAE/gm of dry extract (Table 3).

Total Phenolic content = Chloroform > Ethanol > Ethyl acetate > Petroleum ether

The total flavonoid content was determined by aluminium chloride colorimetric method and the results were expressed as quercetin equivalents. The total flavonoid content found to be high in chloroform extract which showed 428.97 mg quercetin equivalent/gm of extract followed by petroleum ether extract which showed a total flavonoid content of 124.22 mg quercetin equivalent/gm of extract. The total flavonoid content in ethyl acetate extract and ethanol extract was found to be 28.01 and 22.67 mg quercetin equivalent/gm of extract respectively (Table 3).

Total Flavonoid content = Chloroform > Petroleum ether > Ethyl acetate > Ethanol

The total tannin content was found to be high in chloroform extract which showed 80.88 mg tannic acid equivalents per g of dry extract. The total tannin content in ethanol content was found to be 64.38 mg tannic acid equivalents per g of dry extract. Ethyl acetate and petroleum ether extracts showed a tannin content of 48.74 and 15.73 mg tannic acid equivalents per g of dry extract (Table 3).

Total Tannin content = Chloroform > Ethanol > Ethyl acetate > Petroleum ether

Total antioxidant capacity

Total antioxidant capacity of different solvent extracts of *Cichorium intybus* leaves were evaluated by phosphomolybdenum method and the results were expressed as ascorbic acid equivalents. Total antioxidant capacity of petroleum ether extract was found to be 633.30 µg ascorbic acid equivalent per mg of extract,

Chloroform extract showed 1450.59 µg ascorbic acid equivalent per milligram of extract, ethyl acetate extract showed 104.47 µg ascorbic acid equivalent per mg of extract and ethanol extract showed 56.40 µg ascorbic acid equivalent per mg of extract (Table 3).

Total Antioxidant activity = Chloroform > Petroleum ether > Ethyl acetate > Ethanol

Free radical Scavenging activity

The free radical scavenging activity of different solvent extracts of *Cichorium intybus* leaves were investigated using DPPH scavenging activity, nitric oxide scavenging activity, lipid peroxidation scavenging activity, cupric ion reducing assay, ferric reducing assay and metal chelating activity.

DPPH radical scavenging activity

The DPPH radical scavenging activity of various solvent extracts of *Cichorium intybus* leaves is shown in table 4 and fig 6. The ethyl acetate extract showed a significant dose-dependent inhibition of DPPH activity with a 50 % inhibition (IC_{50}) at a concentration of 169.64 µg/ml. The IC_{50} of DPPH radical scavenging activity of ethanol extract was found to be 204 µg/ml, whereas petroleum ether and chloroform extract showed IC_{50} above 1000 µg/ml. As lower IC_{50} values indicate higher radical scavenging activity, ethyl acetate extract showed a potent DPPH radical scavenging activity compared to other solvent extracts.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of various solvent extracts of *Cichorium intybus* leaves is shown in Table 4 and figure 7. It was observed that ethyl acetate extract showed a potent nitric oxide scavenging activity by reducing the amount of nitrite generated from the decomposition of sodium nitroprusside *invitro* with an IC_{50} value of 3.71 µg/ml followed by chloroform and petroleum ether extract with IC_{50} value of 4.30 µg/ml and 5.26 µg/ml respectively. Ethanol extract showed a least nitric oxide scavenging activity with an IC_{50} value of 81.30 µg/ml.

Lipid peroxidation inhibitory activity

The lipid peroxidation inhibitory activity of various solvent extracts of *Cichorium intybus* leaves is shown in table 4 and figure 8. It was observed that ethyl acetate extract showed a potent potent inhibitory effect on untrasound induced lipid peroxidation in liposome prepared from egg lecithin with an IC_{50} value of 9.83 µg/ml followed by chloroform and petroleum ether extract with IC_{50} value of 12.37 µg/ml and 19.55 µg/ml respectively. Ethanol extract showed a least Lipid peroxidation inhibitory activity among the solvent extract with an IC_{50} value of 19.55 µg/ml

CUPRAC assay

CU^{2+} ion reducing capacity of *Cichorium intybus* leaf extract is shown in table 5 and expressed as trolox equivalent. CU^{2+} ion reducing capacity of petroleum ether was found to be 5866.52 µg of trolox equivalent per mg of extract. CU^{2+} ion reducing capacity of chloroform extract was found to be 13398.87 µg of trolox

equivalent per mg of extract. CU^{2+} ion reducing capacity of ethyl acetate extract was found to be 991.617 µg of trolox equivalent per mg of extract and CU^{2+} ion reducing capacity of ethanol extract was found to be 549.439 µg of trolox equivalent per mg of extract.

Ferric reducing power assay

The ferric reducing power of different solvent extracts is shown in fig 9. It was observed that the reducing ability of the extracts increased with the concentration. Among the extracts tested for the reducing abilities, ethanol extract showed a potent reducing power as shown by increased optical density at 700 nm followed by ethyl acetate and chloroform extract.

Metal chelating activity

Metal chelating activity of different extracts of *Cichorium intybus* leaves is shown in fig 10. The chelating of ferrous ion by the extract was estimated in the presence of other chelating agent, ferrozine. It was observed that in the presence of extract, the ferrozine complex formation is disrupted with the result that the red colour of the complex decreases. A dose dependent decrease in absorbance was noted. Among the different solvent extracts, ethanol extract showed a potent metal chelating activity followed by ethyl acetate, petroleum ether and chloroform extract.

From the quantitative estimation of bioactive compounds and free radical scavenging of various solvent extracts of *Cichorium intybus*, it was observed that the chloroform extract was highly potent compared to other extract. Based on the observation, the chloroform extract was selected for in-vivo pharmacological study.

In-vivo pharmacological activity

Acute oral Toxicity study

Acute oral toxicity study was carried out as per OECD guideline 425. From the limit test results it was observed that, the *Cichorium intybus* leaf extract was safe upto a dose level of 2000 mg/kg. There was no mortality and the experimental animals did not show any toxic effect throughout the observation period of 14 days (Table 6).

Effect of *Cichorium intybus* leaf extract on body weight during N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats

Average body weights of different animal groups at various intervals are shown in Figure 11. There was no significant difference in final body weight between the control group (240 ± 2.88 g) and extract alone treated groups (Extract 200 mg/kg- 235.5 ± 2.81 g, Extract 400 mg/kg- 245.2 ± 1.53 g). The final body weight of NDEA induced untreated animals (130.5 ± 3.5 g) was significantly less ($p<0.001$) compared to control animals (240 ± 2.88 g). The group V and group VI animals with NDEA induced and treated with extract 200 mg/kg and 400 mg/kg showed a significant increase ($p<0.001$) in final body weight (Extract 200 mg/kg- 212.6 ± 2.47 g and Extract 400 mg/kg- 225.8 ± 3.0 g) compared to untreated animals. It shows the *Cichorium intybus* leaf extract prevented the body weight loss in NDEA induced animals and maintained the growth rate near normal.

Relative liver weight

The relative liver weight of control and experimental group of animals are shown in table 7 and figure 13. The results were expressed as g liver/100 g body wt. There was no statistical difference in relative liver weight between control (2.22 ± 0.01) and extract alone treated groups (Extract 200 mg/kg - 2.18 ± 0.03, Extract 400 mg/kg - 2.23 ± 0.03). The relative liver weight of group IV NDEA induced untreated animals (2.95 ± 0.06) were significantly increased ($p<0.001$) compared to Group I control animals. A significant decrease ($p<0.001$) in relative liver weight was noted in NDEA induced group V and group VI animals treated with extract 200 mg/kg (2.59 ± 0.05) and 400 mg/kg (2.52 ± 0.04).

Gross morphology

As depicted in figure 12, the appearance of liver in control group rats were normal and there were no macroscopically detectable changes in liver (Fig 12.A). No obvious changes in liver morphology were observed in extract alone treated group which is indicative of nontoxic nature of *Cichorium Intybus* leaf extract (Fig.12B and Fig.12C). The rats in NDEA group revealed enlarged liver and multiple lesions (whitish) with almost the entire surface of liver is occupied with abnormal growth in posterior surface (Fig. 12D). In group of NDEA induced animals treated with *Cichorium Intybus* leaf extract 200 mg/kg, compared to NDEA control there was a marked reduction in damage caused by NDEA on gross examination of anterior and posterior surface. There were no detectable lesions or nodules (Fig. 12E). The liver of rats treated with *Cichorium Intybus* leaf extract 400 mg/kg appeared similar to normal rat liver. Both anterior and posterior surface of liver appeared smooth and there were no detectable lesions or nodules (Fig. 12F).

Effect of *Cichorium intybus* leaf extract on serum marker enzymes and hepatocarcinogenesis marker

The effect of *Cichorium intybus* leaf extract on the activities of marker enzymes and serum protein levels in control and experimental groups are shown in table 8 and figure 14. A significant increase ($P<0.001$) in AST (69.33 ± 0.20), ALT (436.40 ± 10.38), ALP (465.80 ± 9.93) and GGT (56.38 ± 2.35) IU/L were observed in NDEA induced untreated animals compared to control animals with AST (11.47 ± 0.17), ALT (110.0 ± 0.92), ALP (215.3 ± 3.60) and GGT (12.67 ± 0.20) IU/L. The animals treated with *Cichorium intybus* leaf extract 200 mg/kg showed a significant decrease in AST, ALT, ALP and GGT levels of 16.15 ± 0.20 ($p<0.001$), 162.60 ± 3.15 ($p<0.001$), 232.50 ± 6.40 ($p<0.001$) and 23.68 ± 0.90 ($p<0.001$) respectively compared to NDEA untreated animals. The animals treated with *Cichorium intybus* leaf extract 400 mg/kg showed significant decrease in AST, ALT, ALP and GGT levels of 12.17 ± 0.46 ($p<0.001$), 121.80 ± 1.63 ($p<0.001$), 221.5 ± 4.80 ($p<0.001$) and 15.22 ± 0.44 IU/L ($p<0.001$). The total protein levels were decreased ($P<0.001$) in NDEA untreated animals (4.78 ± 0.21) compared to control animals (7.88 ± 0.21). However, upon treatment with *Cichorium intybus* leaf extract 200 mg/kg and 400 mg/kg the protein levels were significantly increased to 5.48 ± 0.17 ($p<0.001$) in animals treated with 200 mg/kg and to6.33 ± 0.16 mg/dl in animals treated with 400 mg/kg ($p<0.001$).

The effect of *Cichorium intybus* leaf extract on AFP and CEA levels in serum of control and experimental groups are shown in table 9 and figure 14. A significant increase in AFP (0.89 ± 0.03 ng/ml) ($p<0.001$) and CEA (5.85 ± 0.14

ng/ml) ($p<0.05$) were observed in NDEA induced untreated animals compared to control animals with AFP and CEA levels 0.52 ± 0.03 and 1.5 ± 0.14 ng/ml respectively. In group of animals treated with *Cichorium intybus* leaf extract, the AFP and CEA levels in serum were significantly reduced to 0.71 ± 0.02 and 4.08 ± 0.24 ng/ml in animals treated with 200 mg/kg ($p<0.001$) compared to NDEA untreated animals. In group of animals treated with *Cichorium intybus* leaf extract 400 mg/kg, the AFP and CEA levels in serum were significantly reduced to 0.59 ± 0.03 and 2.44 ± 0.14 ng/ml compared to NDEA untreated animals. Non-significant alteration in serum marker enzymes and hepatocarcinogenesis marker in extract alone treated group II and group III animals when compared with control group I animals indicates the non-toxic nature of *Cichorium intybus* leaf extract.

Effect of *Cichorium intybus* leaf extract on LPO and levels of antioxidant enzymes in liver

The LPO levels in liver homogenate of control and experimental animals are illustrated in Table 10 and Figure 16. A significant increase ($p<0.001$) in the production of MDA (1.69 ± 0.15) was observed in the group IV NDEA intoxicated untreated animals compared to control animals (1.09 ± 0.08). Administration of *Cichorium intybus* leaf extract showed a significant reduction in LPO as evidenced by a significant fall in MDA levels to 1.08 ± 0.07 ($p<0.01$) and 0.96 ± 0.03 ($p<0.001$) at doses 200 mg/kg and 400 mg/kg respectively.

Table 10 to Fig 16 portrays the activities and the levels of antioxidants in liver of control and experimental animals and the results were expressed as U/mg of protein. A significant decrease ($p<0.001$) in activities of SOD (0.85 ± 0.01) and catalase (0.85 ± 0.11) were noted in group IV NDEA intoxicated untreated animals

compared to control animals with SOD (2.62 ± 0.13) and CAT (1.94 ± 0.11) levels. The levels of non-enzymatic antioxidants GSH (0.91 ± 0.02) were also significantly decreased ($p<0.001$) in liver of group IV untreated animals compared to control animals with GSH (2.26 ± 0.07). However, administration of *Cichorium intybus* leaf extract at 200 mg/kg and 400 mg/kg significantly increased the levels of SOD and CAT to near normal levels. The SOD levels in liver of animals treated with extract 200 mg/kg and 400 mg/kg were significantly increased to 1.78 ± 0.03 and 2.36 ± 0.12 ($p<0.001$) respectively compared to NDEA untreated animals. Extract 200 mg/kg and 400 mg/kg significantly increased the CAT levels to 1.14 ± 0.08 and 1.41 ± 0.09 ($p<0.001$) respectively compared to NDEA induced untreated animals. Similarly, administration of extract 200 mg/kg and 400 mg/kg significantly increased the non-enzymatic antioxidants, GSH to levels 1.74 ± 0.04 and 1.93 ± 0.04 respectively compared to NDEA induced untreated animals.

The extract did not produce any deleterious effect on the antioxidant defense system in normal animals which is evidenced from the non-significant alteration of the enzymatic and non-enzymatic antioxidants along with the maintained rate of lipid peroxidation in group II and group III animals when compared with the normal control group I animals. The results indicate that, the level of lipid peroxidation which increased in liver of carcinogen administered animals was lowered in extract treated animals and in contrast the antioxidant status which was found to be decreased in carcinogen administered animals was improved to near normal upon *Cichorium intybus* leaf extract administration. This indicates that *Cichorium intybus* leaf extract contributes to exert antioxidant defense mechanism.

Histopathology

Histopathological examination of liver sections from control group animals revealed normal architecture (Fig. 17 A). The liver sections of group II and group III animals given extract alone for 16 weeks showed normal architecture. The portal tracts were normal. The hepatic parenchyma showed normal hepatocytes. The central vein and sinusoids appeared normal. There is no evidence of inflammation or malignancy or atypical cells depicting the non-toxic nature of *Cichorium intybus* leaf extract (Fig. 17 B and Fig. 17 C). Section of liver from group IV NDEA induced untreated animals revealed loss of architecture with portal tract inflammation with infiltration of lymphocytes (Fig. 17 D). In contrast, NDEA induced animals treated with *Cichorium intybus* leaf extract 200 mg/kg showed chronic portal tract inflammation with periportal macrovesicular steatosis (Fig. 17 E), whereas the liver sections of NDEA induced animals treated with *Cichorium intybus* leaf extract 400 mg/kg showed normal hepatic architecture (Fig. 17 F). The portal tracts were normal and the hepatic parenchyma showed normal hepatocytes with no evidence of cirrhosis (40×, HE).

DISCUSSION

Liver cancer, one of the most common cancer worldwide, accounts approximately 549,000 deaths each year (Liu *et al.,* 2006). As liver, the major metabolic organ in our body is responsible for production of ROS during metabolism of carcinogens might be responsible for carcinogenic effects. N-nitrosodiethylamine (NDEA), one of the most important environmental carcinogen on metabolic transformation in liver produces ROS that act as promutagenic products is responsible for carcinogenic effects (Bansal *et al.* 2005). Therefore, development of a compound with potent antioxidant activity can prevent the formation of ROS and thus provide protection against the deteriorating outcome. Traditionally many herbal medicines have revealed cancer chemopreventive potential through antioxidant activity. The present study was aimed to evaluate the chemopreventive and antioxidant activity of chloroform extract of *Cichorium intybus* leaves against N-nitrosodiethylamine induced hepatocarcinogenesis. As a preliminary study, the radical scavenging and invitro antioxidant activity of different solvent extracts of *Cichorium intybus* Linn.leaves were investgated. The preliminary phytochemical screening showed the presence of phenolics, tannins and flavonoids which are the major constituents of antioxidant activity, so it is dual necessary to estimate the bioactive compounds in various solvent extracts. Highest content of phenolic, tannins and flavonoids were noted in chloroform extract in comparison with other solvent used in quantitative analysis. The increased polarity made this organic solvent an ideal and selective to extract a great number of bioactive phenolic compounds. This indicated that the highest total antioxidant activity observed in

chloroform extract of *Cichorium intybus* leaves is also due to the presence of high phenolics, tannins and flavonoids content. The radical scavenging and antioxidant activity of solvent extracts were demonstrated using different in vitro bioanalytical methodologies. Various antioxidant assay methods have been developed to identify antioxidant capacity.

DPPH radical scavenging activity is the oldest method for determining the antioxidant activity. DPPH is astable nitrogen centered free radical which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption. DPPH is a purple colour dye having absorption maximal of 517 nm and upon reaction with hydrogen donar the purple colour fades or disappears due to conversion of it to 2,2-diphenyl-1-picryl hydrazine resulting in decrease in absorbance (Kumarasamy *et al.,* 2007). Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers. In the present study, it was found that the radical scavenging activity of chloroform extract of *Cichorium intybus* leaves is relatively high compared to other solvent extracts. This might be due to the H^+ donating capacity due to the significant presence of flavonoids and phenolic compounds.

Nitric oxide is an important regulatory molecule involved in the regulation of various physiological functions such as neurotransmission, vascular homeostasis, inhibition of platelet aggregation, regulation of cell mediated toxicity and importantly for host defense. It acts as a signal molecule in immune, nervous and vascular systems. However, excess production of nitric oxide is associated with several diseases (Karmakar *et al.,* 2011). The toxicity of nitric oxide increases

greatly when it reacts with superoxide radical, forming highly reactive peroxy nitrite anion ($ONOO^-$). Therefore compounds with potent nitric oxide inhibition will be valuable for therapeutic use (Hepsibha *et al.,* 2010). The principle of the present study is sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that is estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions (Ebrahimzadeh *et al.,* 2009). In the present investigation chloroform extract of *Cichorium intybus* exhibited a potent nitric oxide scavenging activity which might be due to inhibition of nitric oxide formation by directly competing with oxygen in reaction with nitric oxide.

Lipid peroxidation has long been thought to be a toxicological phenomenon that can lead to various pathological consequences (Hochstein and Atallah, 1998). ROS, such as hydroxyl radical initiate's lipid peroxidation by extracting a hydrogen atom from lipids forming conjugated lipid radical. This reacts rapidly with oxygen to form a lipid radical, until chain reaction is terminated. The lipid peroxidation adducts induce the oxidation of biomolecules such as DNA, proteins and other lipids resulting in cellular damage (Resat *et al.* 2007; Huong *et al.,* 1998). Since lipid peroxidation is found to be important cause of cell membrane destruction and cell damage, their detoxification appears to be critical for the survival of an organism in oxidative stress (Dargel, 1992). Therefore antioxidants play a vital role in inhibition of lipid peroxidation or protection against cellular damage by free radicals. It is reported that phenolic and flavonoids are powerful antioxidants which can scavenge lipid free radical. In the present study, the high peroxidation scavenging effect of chloroform extract of *Cichorium intybus* leaves might be due to the presence of

phenolic and flavonoid content which can terminate peroxidation chain reaction easily and quench reactive oxygen and nitrogen species, thereby inhibiting the oxidation of lipid and other biological molecules.

Reducing power is associated with antioxidant activity and may serve as a significant indicator of potential antioxidant activity (Gulcin, 2010). Compounds which are electron donars can reduce the oxidised intermediates of lipid peroxidation processes, which in turn act as primary and secondary antioxidants (Yen and Chen, 1995). In the present study ferric reducing antioxidant capacity and cupric reducing antioxidant capacity (CUPRAC) were used for the measurement of reducing capacity of extract. Both the methods are based on electron transfer and are considered as good indicator for total antioxidant power (Tezcan *et al.,* 2011). Ferric reducing antioxidant capacity is based on reduction of Fe^{3+} to Fe^{2+} by antioxidants. CUPRAC assay is based on reduction of Cu^{2+} to Cu^{+} by antioxidants. This method is suitable for variety of antioxidants regardless of chemical type or hydrophilicity. Moreover, the results obtained from invitro cupric ion (Cu^{2+}) reducing measurements might be more efficiently extended to the possible invivo reactions of antioxidants (Gulcin and Dastan, 2007; Karaman *et al.,* 2010). In the present study, chloroform extract exhibited the most reducing power and by correlating the results with quantitative analysis, the reducing power of extract might be due to the presence of high phenolic content because phenolic compounds usually show high reducing capacity.

As a catalyst of oxidative process, bivalent transition metal ions play an important role in the formation of hydroxyl radical and hydroperoxide decomposition reactions (Halliwell, 1997). Peroxides which are capable of

generating free radicals may be implicated in human cardiovascular diseases (Halliwell and Gutteridge, 1990). Because of Fe^{2+} causes the production of oxyradicals and lipid peroxidation, minimizing its concentration affords protection against oxidative damage. Chelating agents which form complex with metal ion are found to be effective in reducing the redox potential and thereby stabilizing the oxidized form of metal ion (Gordon, 1990). In the present study, the chloroform extract of *Cichorium intybus* leaves efficiently interfered the formation of ferrous and ferrozine complex, suggesting that the ethanol extract of *Cichorium intybus* leaves has good chelating effect and is able to capture ferrous ion before ferrozine.

Dose levels for *in-vivo* pharmacological screening were selected based on the acute oral toxicity study and our previous *in-vivo* studies, where the extract showed protection against CCl_4 induced hepatotoxicity in dose levels of 250 mg/kg and 500 mg/kg. In the present study two dose levels $1/10^{th}$ and $1/5^{th}$ of 2000 mg/kg were selected from the acute toxicity study, where the extract was found to be safe up to a dose level of 2000 mg/kg.

Decrease in body weight observed during the experimental period in NDEA induced untreated animals could be largely due to deterioration of liver function and nutritional deprivation which might be due to reduced food intake. In addition, assessment of relative liver weight was used as potential tool to diagnose change in liver size. NDEA induced proliferation of cells in the liver was evident from increase in NDEA induced untreated animals. Treatment with *Cichorium intybus* leaf extract prevented body weight loss and reduced the relative liver weight compared to untreated animals, which signify the amelioration capacity of extract upon carcinogen exposure. Significant decrease in lesions in liver of treatment

groups were supported by histological assessment of liver. The pathological changes in liver caused by NDEA were assessed by determining the levels of various biochemical hepatic markers (Kovalsky *et al.,* 1996). Marked elevation in AST and ALT in NDEA induced untreated animals indicated the hepatocellular damage. Because AST and ALT are cytoplasmic in location, they are released into circulation after cellular damage and rupture of plasma membrane (Wroblewski, 1959; Sallie *et al.,* 1951). Hence AST and ALT are considered as sensitive markers employed in diagnosis of hepatic damage. Treatment with *Cichorium intybus* leaf extract significantly reduced the levels of AST and ALT in NDEA induced animals. This indicates that *Cichorium intybus* leaf extract tends to prevent liver damage by maintaining integrity of plasma membrane, thereby suppressing the leakage of enzymes through membrane.

The elevation in ALP in NDEA induced untreated animals might be due to proliferation of bile ductular cells and resulting blockade of bile ducts. Significant reduction in ALP levels in NDEA induced extract treated animals indicates the protective effect of *Cichorium intybus* leaf extract against NDEA induced hepatic injury. The reduction in serum protein levels in NDEA induced untreated animals might be due to liver damage because hepatotoxicity impairs the synthetic function of liver (David, 1999). Treatment with *Cichorium intybus* leaf extract ameliorated the imbalance.

GGT, an enzyme embedded in plasma membrane of hepatocytes, mainly in canalicular domain. Any damage in plasma membrane causes liberation of GGT into serum (Sivaramakrishnan *et al.,* 2008). Hence, GGT is considered as a best indicator of liver damage (Bulle *et al.,* 1990). Elevated levels of GGT observed in NDEA

induced untreated animals might be due to liver injury. Treatment with *Cichorium intybus* leaf extract significantly lowered the GGT level which indicates that *Cichorium intybus* leaf extract tends to prevent liver damage by maintaining integrity of plasma membrane.

A tumour associated foetal protein, AFP has long been employed as a serum tumour marker to monitor disease progression (Abelev, 1971). AFP is a 72 KDa α_1 globulin synthesized during embryonic life by foetal yolk sac, liver and intestinal tract with uncertain biological function. Since AFP has high specificity for hepatocarcinoma (Liu *et al.*, 2006), its serum concentration can be used in diagnosis of hepatocarcinoma and for the diagnosis of tumour response to therapy.

CEA is a glycoprotein found in different cells, but typically associated with certain tumours. Increase in CEA levels is most frequent in cancer of colon and rectum. The conditions which can elevate CEA are smoking, pancreatitis, inflammatory bowel disease, pancreatitis and cirrhosis of liver. CEA levels increase, with an increase in tumour size (Jahan *et al.,* 2011). In the present study, significant increase in AFP and CEA levels observed in NDEA induced untreated animals might have caused due to mutagenesis. The decrease in levels of AFP and CEA following treatment with *Cichorium intybus* leaf might be due to response to therapy.

Increased ROS generation and decreased antioxidant defense in liver tissue has been reported in development of hepatocarcinogenesis (Ramakrishnan *et al.,* 2006; Kweon *et al.,* 2003). ROS, through interaction with nucleic acids, proteins, lipids results in chromosomal instability, mutations, loss of organelle function play an important role in development of cancer (Waris and Ahsan, 2006). Lipid

peroxidation is one of the major mechanisms of cell injury caused by free radicals. Enormous amount of free radicals generated by carcinogens reacts with lipids causing lipid peroxidation (Sikkim and Mulee 2000). The products of lipid peroxidation include malondialdehyde that interacts with various molecules leading to oxidative stress and has been reported to be involved in the formation of tumours (Ramakrishnan *et al.,* 2007). In the present study, significant increase in the levels of lipid peroxidation observed in untreated group IV cancer bearing animals may be due to the excessive production of free radicals and due to inhibition of antioxidant enzymes. Significantly reduced levels of lipid peroxidation were seen in *Cichorium intybus* leaf extract treated group V and group VI NDEA induced animals. This clearly shows that *Cichorium intybus* effectively controlled the rate of lipid peroxidation, which suggests the beneficial effect of the extract against NDEA initiated free radical formation. The presence of flavonoids may contribute to this effect, because they are proved to be a potential inhibitor of lipid peroxidation (Siegers and Younes, 1981).

The enhanced formation of lipid peroxides is further evidenced by decrease in activities of antioxidant enzymes in liver of NDEA induced untreated animals as compared with normal control animals.

SOD is said to act as the first line of defense against superoxide radical generated as a by-product of oxidative phosphorylation. SOD mediated dismutation of superoxide radical (O_2^-) generates hydrogen peroxide (H_2O_2). Accumulation of excess of H_2O_2 causes toxic effects on cellular system. In this regard catalase converts H_2O_2 into water (Li Shijun *et al.,* 2000). CAT detoxifies H_2O_2 into H_2O and O_2 (Murray *et al.*, 2003). Thus SOD and act mutually and constitute the enzymatic

defense mechanism against ROS (Bhattacharjee and Chatterjee, 1998). In the present study decrease in the activities of SOD and CAT in untreated cancer bearing animals could be attributed to excessive utilization of enzymes in detoxification of peroxides and hydroperoxides generated during liver carcinogenesis. Restoration in the levels of lipid peroxidation upon treatment with *Cichorium intybus* leaf extract might have resulted in the recoupment in the activities of the above antioxidant enzymes to normalcy.

GSH is a well known non-enzymatic antioxidant defense system of cells, act synergistically to scavenge free radicals in biological system. GSH is found to be present in high concentration in cells, protects cells against free radical attack (Farombi *et al.,* 2000). GSH acts directly as free radical scavenger by donating a hydrogen atom and thereby neutralizing hydroxyl radical. It reduces peroxides and maintains protein thiols in the reduced state (Nwanjo and Oze, 2007). GSH-P_X uses GSH as a substrate to catalyze the reduction of hydroperoxide and H_2O_2 (Bebe and Panemangalore, 2003). Reduced glutathione (GSH) in tissues maintains maintains the cellular levels of vitamin C and Vitamin E in active form. It removes free radicals from cytosol and plays a vital role in protecting lipoprotein molecules from oxidative damage by regenerating the reduced form of vitamin E (Das, 1994). GSH acts synergistically with vitamin E against oxidative stress (Chaudiere, 1994). Vitamin C also scavenges and detoxifies free radicals in combination with Vitamin E and GSH (George, 2003). The decreased level of GSH observed in NDEA induced untreated animals might be due to excessive utilization of these antioxidants for quenching enormous free radicals produced. Treatment with *Cichorium intybus* leaf extract effectively restored the depleted levels of non-enzymatic antioxidants

GSH caused by NDEA. Increase in GSH levels in turn also contributes to the recycling of other antioxidants such as vitamin C and vitamin E.

The results indicate that *Cichorium intybus* leaf extract inhibits the level of lipid peroxidation and significantly increases the enzymatic and non-enzymatic antioxidant defense mechanisms in NDEA induced liver carcinogenesis.

The histological observations clearly indicate the presence of cirrhotic changes in NDEA induced untreated animals. In animals treated with *Cichorium intybus* leaf extract the liver architecture was preserved which clearly supports the biochemical data. These suggest the *Cichorium intybus* leaves might have promising protective role against liver carcinogenesis.

CONCLUSION

This study suggest that administration of chloroform extract of *Cichorium intybus* leaves possesses a significant protection against NDEA induced liver carcinogenesis by a mechanism related, at least in part, to the ability of *Cichorium intybus* leaves to decrease oxidative stress by stabilizing and increasing all the components of antioxidant defense system which were disturbed system during NDEA induced oxidative stress in rats. The chloroform extract of *Cichorium intybus* leaves was found to be rich in flavonoids, phenolics and tannins and showed a potent free radical scavenging activity screening in in-vitro analysis. Since flavonoids, phenolics and tannins are powerful antioxidants, their presence in *Cichorium intybus* leaf extract might be responsible for antioxidant property, which might be involved in the chemoprevention against hepatocarcinogenesis.

BIBLIOGRAPHY

1. Abelev GI. Alpha-feto protein in association with malignant tumours. *Adv Cancer Res* 1971; 14: 295-357.
2. Ahmed B, Al-Howiriny TA, Siddiqui AB. Antihepatotoxic activity of seeds of *cichorium intybus*. *J. Ethnopharmacol* 2003; 87: 237-240.
3. Akintonwa DA. The derivation of nitrosamines from some therapeutic amines in the human environment. *Ecotoxicol Environ Saf* 1985; 9: 64–70.
4. Ali WS. Antihyperglycaemic effect of cichory leaves and vanadium consumption on diabetic experimental rats. *World J. Dairy & Food Sci* 2012; 7(2): 167-173.
5. Apak R, Guçlu K, Ozyurek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC Method. *J Agric Food Chem* 2004; 52: 7970- 7981.
6. Atta AH, Elkoly TA, Mouneir SM, Alwabel NA *et al.* Hepatoprotective effect of methanol extracts of zingiber officinale and *Cichorium intybus. Indian J Pharm sci* 2010; 72(5): 564-70.
7. Bais HP, Ravishankar GA. Cichorium intybus L – cultivation, processing, utility, value addition and biotechnology, with an emphasis on current status and future prospects. *Journal of the Science of Food and Agriculture* 2001; 81: 467-484.

8. Bansal AK, Trivedi R, Soni GL, Bhatnagar D. Protective role of vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver. *Chem Biol Interact* 2005; 156: 101-111.

9. Bebe FN, Panemangalore M. Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissues of rats. *J Environ Sci Health* 2003; B38: 349-363.

10. Befeler AS, Di Bisceglie AM. Hepatocellular carcinoma: diagnosis and treatment. *Gasteroenterology* 2002; 122 (6): 1609-1619.

11. Bhattracharya S, Chatterjee M. Protective role of *Trianthema portulacastrum* against diethylnitrosamine- induced experimental hepatocarcinogenesis. *Cancer Lett* 1998; 129:7-13.

12. Bhosale P, Motiwale L, Ignle AD, Gadre RV, Rao KVK Protective effect of *Rhodotorula glutinis* NCIM3353 on the development of hepatic preneoplastic lesions. *Curr Sci* 2002; 83 (3): 303–308.

13. Bing L, Yun Q. The pharmacology research progression of Gly acid and gly-cyrrhetic acid, foreign medicine. *Planta Med* 2006; 21: 100-104.

14. Bishayee A, Dhir N. Resveratrol-mediated chemoprevention of diethylnitrosamine-initiated hepatocarcinogenesis: inhibition of cell proliferation and induction of apoptosis. *Chem Biol Interact* 2009; 179 (2–3): 131–144.

15. Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature* 1958; 181: 1199-1200.

16. Bulle F, Mavier P, Zafrani ES, Preaux AM, Lescs MC, Siegrist S, Dhumeaux D, Guellaen G. Mechanism of gamma-glutamyl transpeptidase release in

serum during intra hepatic and extra hepatic cholestasis in the rat: a histochemical, biochemical and molecular approach. *Hepatology* 1990; 11: 545-550.

17. Chang C, Yang M, Wen H, Chen J. Estimation of total flavonoids content in propolis by two complementary colorimetric methods. *J Food Drug Anal* 2002; 10: 178–182.
18. Chaplin DJ, Pettit GR, Hill SA. Anti-vascular approaches to solid tumor therapy: evaluation of combretastatin A4 phosphate. *Anticancer Res* 1999; 19: 189- 195.
19. Chaudiere J. Some chemical and biochemical constrains of oxidative stress in living cells. In: Rice-Evans CA, Burdon RH (Eds), Free radical damage and its control. *Elsevier Science*, *Amsterdam* 1994; 25-26.
20. Chhaya G, Mishra SH. Antihepatotoxic activity of *Cichorium intybus*. *Journal of Ethnopharmacology* 1997; 58: 131–134.
21. Cho YJ, Sinha J, Park JP and Yun JW. Production of inulooligosaccharides from chicory extract by endoinulinase from Xanthomonas oryzae *enzyme. Microb Technol* 3-8-2001; 28 (4-5):439-445.
22. Chopra RN, Chopra IC, Hand KL. Indigenous Drugs of India 1996; Academic Publishers, Calcutta.
23. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology* 2005; 100: 72–79.
24. Cragg GM, Newman DJ. Plants as a source of anti-cancer and anti-HIV agents. *Ann. appl. Biol* 2003; 143:127-133.
25. Dargel R. Lipid peroxidation - a common pathogenetic mechanism. *Exp Toxicol Pathol* 1992; 44: 169-181.

26. Das S. Vitamin E in the genesis and prevention of cancer. A review. *Acta Oncol* 1994; 33: 615–619.

27. David EJ. Special considerations in interpreting liver function tests. *Am Family Phy* 1999; 59: 8.

28. De Kraker JW, Franssen MC, De Groot A, Konig WA *et al*. (+)-Germacrene A biosynthesis. The committed step in the biosynthesis of bitter sesquiterpene lactones in chicory. *Plant Physiol* 1998; 117 (4):1381-1392.

29. Dinis TCP, Madeira VMC, Almeida MLM. Action of phenolic derivates (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Arch Biochem Biophys* 1994; 315: 161-169.

30. Du H, Yuan S, and Jiang P. [Chemical constituents of Cichorium intybus L.]. *Zhongguo Zhong Yao Za Zhi* 1998; 23(11): 682-3, 704.

31. Duh PD, Yen GH. Antioxidative activity of three herbal water extracts. *Food chemistry* 1997; 60: 639-645.

32. Ebrahimzadeh MA, Nabavi SF and Nabavi SM. Antioxidant activities of methanol extract of *Sambucus ebulus* L. flower. *Pak J Biol Sci* 2009; 12(5): 447-450.

33. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70–77.

34. Farombi EO, Olowg BI, Emerole GO. Effect of three structurally related antimalarial drugs on liver microsomal components and lipid peroxidation in rats. *Comp Biochem Physiol* 2000; 126: 217-224.

35. Feo F, Pascale RM, Simile MM, De Miglio MR, Muroni MR, Calvisi D. Genetic alterations in liver carcinogensis; implications for new preventive and therapeutic strategies. *Crit Rev Oncog* 2000; 11: 19–62.

36. Finke B, Stahl B, Pritschet M, Facius D *et al.* Preparative continuous annular chromatography (P-CAC) enables the large-scale fractionation of fructans. *J Agric Food Chem* 2002; 50(17): 4743-4748.

37. Folch J, Lees M, Solane SGH. A simple method for isolation and purification of total lipids from animal tissues. *J Bio Chem* 1957; 26: 497–509.

38. George J. Ascorbic acid concentrations in diethylnitrosamine induced hepatic fibrosis in rats. *Clin Chem Acta* 2003; 335: 3.

39. Gordon MH. The mechanism of antioxidant action in vitro. *In*: Hudson BJF (editor). Food antioxidants. *Elsevier Applied Science. London,* 1990, pp.1-18.

40. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR. Analysis of nitrate, nitrite and (15N) nitrate in biological fluids. *Anal Biochem* 1982; 126: 131-138.

41. Gulcin I, Dastan A. Synthesis of dimeric phenol derivatives and determination of invitro antioxidant and radical scavenging activities. *J Enzym Inhib Med Chem* 2007; 22: 685-695.

42. Gulcin I. Antioxidant properties of resveratrol: a structure–activity insight. *Innov Food Sci Emerg* 2010; 11: 210–218.

43. Gupta LM and Raina R. Side effects of some medicinal plants. *Current Science* 1998; 75: 897-900.

44. Hai W, Kim C, Song S, Kang C. Study on mechanism of multistep hepatotumorigenesis in rat: development of hepatotumorigenesis. *J Vet Sci* 2001; 2: 53–58.

45. Halim AF, Collins. Essential oil analysis of Myricaceae of Eastern United States. *Phytochemistry* 1973; 12: 1077–1083.

46. Halliwell B and Gutteridge JMC. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol* 1990; 186: 1-85.

47. Halliwell B. Antioxidants: the basics - what they are and how to evaluate them. *Adv Pharmacol* 1997; 38: 3-20.

48. Halsted CH, Villanueva J, Chandler CJ, Stabler SP, Allen RH, Muskhelishvili L, James SJ, Poirier L. Ethanol feeding of micropigs alters methionine Metabolism and increases hepatocellular apoptosis and proliferation. *Hepatology* 1996; 23 (3): 497–505.

49. Häring DA, Maurer V, Senn M, Hertzberg H. Individual administration of three tanniferous forage plants to lambs artificially infected with Haemonchus contortus and Cooperia curticei. *Vet Parasito* 2007; 146 (1–2): 123–34.

50. He Y, Guo YJ and Gao YY. Studies on chemical constituents of root of Cichorium intybus. *Zhongguo Zhong Yao Za Zhi* 2002; 27(3): 209-210.

51. Heibatollah S, Reza NM, Izadpanah G, Sohailla S. Hepatoprotective effect of *Cichorium intybus* on CCl4 induced liver damage in rats. *Afr. J. Biochem. Res* 2008; 2(6): 141-144.

52. Helal EGE, Abd EI-Wahab SM, Moussa Sharaf AM, Zedan GA. Effect of *Cichorium intybus L.* on fatty liver induced by oxytetracycline in albino rats. *The Egyptian journals of hospital medicine* 2011; 45: 522-535.

53. Hepsibha BT, Sathiya S, Babu CS, Premalakshmi V, Sekar T. Invitro studies of antioxidant and free radical scavenging activities of *Azima tetracantha* Lam leaf extract. *Indian J Sci Technol* 2010; 3: 571-577.

54. Hochstein P, Atallah AS. The nature of antioxidants and antioxidant systems in the inhibition of mutation and cancer. *Mutat Res* 1998; 202: 363-375.

55. Huong NTT, Malsumato K, Kasai R, Yamasaki K, Watanabeth. Invitro antioxidant activity of Vietnamese ginseng saponin and its components. *Biol Pharm Bull* 1998; 21: 978-981.

56. IARC Monograph on the evaluation of carcinogenic risk of chemicals to man, vol.1 *International Agency for Research on Cancer*, *Lyon* 1972; 107–124.

57. Isbrucker RA, Burdock GA. Risk and safety assessment on the consumption of Licorice root (*Glycyrrhiza sp.*), its extract and powder as a food ingredient, with emphasis on the pharmacology and toxicology of Gly. Regul. Toxicol. *Pharmacol*. 2006; 46: 167-192.

58. Jagadeeswaran R. Thirunavukkarasu C, Nalini R, Gunasekaran S, Sakthisekaran D In vitro studies on the selective cytotoxic effect of crocetin and quercetin. *Fitotherapia* 2000; 71(4): 395-399.

59. Jahan MS, Vani G, Shyamaladevi CS. Anti-carcinogenic effect of *Solanum trilobatum* in diethylnitrosamine induced and Phenobarbital promoted hepatocarcinogenesis in rats. *Asian J Biochem* 2011; 6(1): 74-81.

60. Jamshidzadeh A, Kkhoshnood MJ, Dehghani Z, Niknahad H. Hepatoprotective activity of *Cichorium intybus* L. leaves extract against carbon tetrachloride induced toxicity. *Iran. J. Pharm*. Res 2006; 1: 1-46.

61. Jindal MN, Patel VK, Patel NB. Pharmacological actions of aqueous and alcoholic extracts of roots of *Cichorium intybus* Linn. *Indian Journal of Pharmacology* 1975; 7: 24–33.

62. Jurgonski A, Milala J, Juskiewicz J, Zdunczyk Z *et al.* Composition of cichory root, peel, seed, and leaf ethanol extracts and biological properties of their non-inulin fraction. *Food technol Biotechnol* 2011; 49(1): 40-47.

63. Kakkar P, Das B, Viswanath PN. Modified spectrophotometer assay of SOD. *Ind J Biochem Biophys* 1984; 95: 51–58.

64. Kamboj VP. Herbal Medicine. *Current Science 2000;* 78: 35-39.

65. Karaman S, Tutem E, Baskan KS, Apak R. Comparison of total antioxidant capacity and phenolic composition of some apple juices with combined HPLC-CUPRAC assay. *Food Chem* 2010; 120: 1201-1209.

66. Karmakar I, Dolai N, Saha P, Sarkar N, Bala A, Haldar PK. Scavenging activity of *Curcuma caesia* rhizome against reactive oxygen and nitrogen species. *Orien Pharm Exp Med* 2011; 11: 221-228.

67. Kim J, Park EJ. Cytotoxic anticancer candidates from natural resources. *Curr Med Chem* Anti-cancer Agents 2000; 2: 485-537.

68. Kim JH, Ja Mun Y, Hong woo w, Soo Jeon K *et al.* Effects of the ethanol extract of *cichorium intybus* on the immunotoxicity by ethanol in mice. *Int Immunopharmacol* 2002; 2 : 733- 744.

69. Kimura M, Moro T, Motegi H. In vivo Gly accelerates liver regeneration and rapidly lowers serum transaminase activities in 70% partially hepatectomized rats. *Eur J Pharmacol* 2008; 579: 357-364.

70. Kind PRN, King EJJ. Estimation of plasma phosphatase by determination of hydrolysed phenol with antipyrine. *J Clin Pathol* 1954; 7: 322-330.

71. Kocsis I, Hagymasi K, Kery A, Szoke E, Blazovics A *et al.* Effects of cichory on pancreas status of rats in experimental dislipidemia. *Acta Biologica Szegediensis* 2003; 47(1-4): 143-146.

72. Kokate CK. Practical Pharmacognosy. 4th ed. New Delhi Vallabah Prakashan; 1994; 107-11.

73. Kovalsky I, Schaff Lapis K, Jeney A. Marker enzymes of rat chemical hepatocarcinogenesis in human liver tumours. *Pathol Oncol Res* 1996; 2: 54-55.

74. Kumarasamy Y, Byres M, Cox PJ, Jaspars M, Nahar L, Sarker SD. Screening seeds of some Scottish plants for free-radical scavenging activity. *Phytother Res* 2007; 21(7): 615-621.

75. Kurisu S, Inoue I, Kawagoe T *et al.* Clinical profile of patients with symptomatic Gly-induced hypokalemia. *J. Am. Geriatr. Soc*. 2008; 56: 1579-1581.

76. Kweon S, Park KA, Choi H. Chemopreventive effect of garlic powder diet in diethylnitrosamine-induced rat hepatocarcinogenesis. *Life Sci* 2003; 73: 2515-2526.

77. Li Shijun, Yan Tao, Ji-Qin Yang, Terry DO and Larry WO. The role of cellular glutathione peroxidase redox regulation in the suppression of tumour cell growth by manganese superoxide dismutase. *Cancer Res* 2000; 60: 3927-3939.

78. Liao DJ, Blanck A, Eneroth P, Gustafsson JA, Hällström IP, Diethylnitrosamine causes pituitary damage, disturbs hormone levels and reduces sexual dimorphism of certain liver functions in the rat. *Environ Health Perspect* 2001; 109: 943–947.

79. Liu JG, Zhao HJ, Liu YJ Wang XL. Effect of selenium-enriched malt on hepatocarcinogenesis, paraneoplastic syndrome and the hormones regulating blood glucose in rats treated by diethylitrosamine. *Life Sci* 2006; 78 (20): 2315-2321.

80. Loeppky RN. Nitrosamine and nitroso compound chemistry and biochemistry, in: ACS Symposium Series 553, *American Chemical Society, Washington, DC* 1994; 1–12.

81. Lu P, Luo HS, Zhou XP, Paul SC *et al*. Thalidomide prevents rat liver cirrhosis via inhibition of oxidative stress. *Pathol Res Pract* 2006; 202: 777-788.

82. Ma LS, Wen Y, Zhan *et al.* Anticancer effects of the Chinese medicine Mat on murine hepatocellular carcinoma cells. *Planta Med* 2008; 74: 245-251.

83. Macnab GM, Urbanowicz JM, Kew MC. Carcinoembryonic antigen in hepatocellular cancer. *Br J Cancer* 1978; 38: 51-54.

84. Malloy HT, Evelyn KA. The determination of bilirubin with the photometric colorimeter. *J Biol Chem* 1937; 119: 481-490.

85. Mann J. Natural products in cancer chemotherapy: Past, present and future. *Nat Rev Cancer* 2002; 2: 143-148.

86. Michalopoulos GK, De Frances M. Liver degeneration. *Science* 1997; 276: 60-66.

87. Milala J, Grzelak K, Krol B, Juskiewicz J *et al*. Composition and properties of cichory extracts rich in fructants and polyphenols. *Pol. J. Food Nutr. Sci* 2009; 59(1): 35-43.

88. Moertel CG, Schutt AJ, Reitemeier RJ *et al.* Phase II study of camptothecin (NSC- 100880) in the treatment of advanced gastrointestinal cancer, *Cancer Chemother Rep* 1972; 56: 95-101.

89. Murray RK, Granner DK, Mayes PA. Rodwell VW. Harper's Illustrated Biochemistry, 26th ed, *The McGraw-Hill Companies Inc.*2003.

90. Najafzadeh H, Ghadrdan AR, Jalali M, Alizadeh F. Evaluation of changes of factors related to liver function in serum of horse by administration of *Cichorium intybus. J.Animal veterinary Adv*.2011; 3(1): 1-5.

91. Nandagopal S, Kumari BD. Phytochemical and antibacterial studies of cichory *Advan. Biol. Res.* 2007; 1(1-2): 17-21.

92. Naseem N, Latif MSZ, Tahir M, Naveed AK *et al.* Hepatoprotective effect of *Cichorium intybus* linn extracts against carbon tetra chloride induced liver damage. *J Rawal Med Coll* 2009; 13(2): 53-55.

93. Nehal S, Belal M. Leaves and barley grains to hepatoprotective effect of feeding celery leaves mixed cichory hypercholesterolemic rats. *Asian J clin nutr* 2011; 3(1): 14.

94. Nwanjo HU, Oze GO. Oxidative imbalance and non-enzymic antioxidant status in pulmonary tuberculosis infected. *Pak J Nutr* 2007; 6: 590-592.

95. O'Dwyer PJ, Leyland-Jones B, Alonso MT, Marsoni S *et al*. Current status of an active cancer drug, *N. Engl. J. Med* 1985; 312: 692 – 700.

96. OECD Guidance document on acute oral toxicity 425. Environmental health and safety monograph series on testing assessment 2000; 24.

97. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358.

98. Okugawa H, Ueda R, Matsumoto K, Kawanishi K *et al.* Effect of dehydrocostus lactone and costunolide from Saussurea root on the central nervous system in mice, *Phytomedicine* 1996; 147-153.

99. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Jpn J Nutr* 1986; 44: 307–315.

100. Parola M, Robino G. Oxidative stress-related molecules and liver fibrosis. *J. Hepatol* 2001; 35: 297–306.

101. Patel VK, Bhatt HV. Toxicity and antiseptic effects of chicory root extracts in Pyorrhea. *The Antiseptic* 1985; 904–906.

102. Pettit G R, Singh SB, Cragg G M. Synthesis of natural (-) combretastatin. *J. Org Chem* 1985; 50: 3404- 3406.

103. Pillai TG, Damodhran DP. Prevention of radiation – induced chromosome damage in mouse bone marrow by aqueous leaf extract of *Cichorium intybus. Journal of cell and molecular biology* 2007; 6(1): 59-64.

104. Polshettiwar SA, Ganjiwale RO, Wadher SJ, Yeole PG. Spectrophotometric estimation of total tannins in some ayurvedic eye drops. *Indian J Pharma Sci* 2007; 69: 574-576.

105. Prieto P, Pineda M and Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a Phosphomolybdenum Complex: Specific application to the determination of vitamin *E. Anal. Biochem* 1999; 269: 337-341.

106. Pushparaj PN, Low HK, Manikandan J, Tan BKH, Tan CH. Antidiabetic effects of Cichorium intybus in streptozotocin-induced diabetic rats. *J. Ethnopharmacol* 2007; 111:430-434.

107. Ramakrishnan G, Augustine TA, Jagan S, Vinodhkumar R, Devaki T. Effect of silymarin on N-nitrosodiethylamine induced hepatocarcinogenesis in rats. *Exp Oncol* 2007; 29 (1): 39-44.

108. Ramakrishnan G, Ragahavendran HR, Vinodkumar R, Devaki T. Suppression of N-nitrosodiethylamine induced hepatocarcinogenesis by silymarin in rats. *Chem Biol Interact* 2006; 161: 104-114.

109. Redinbo MR, Stewart L, Kuhn P, Champoux JJ, Hol WGJ. A model for the *mechanism of human topoisomerase I. Science* 1998; 279: 1504-13.

110. Reitman S, Frankel SA. Coloirmetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; 28: 56-63.

111. Resat A, Kubilay G, Birsen D. Comparitive evaluation of various total antioxidant assays applied to phenolic components with the CURAC assay. *Mol* 2007; 12: 1496-1547.

112. Roberfroid MB, Cumps J, Devogelaer JP. "Dietary chicory inulin increases whole-body bone mineral density in growing male rats". *The Journal of nutrition* 2002; 132 (12): 3599–602.

113. Rodgers EH, Grant MH. The effect of the flavonoids, quercetin, myricetin and epicatechin on the growth and enzyme activities of MCF7 human breast cells. *Chem-Biol Interact* 1998; 116: 213–218.

114. Rosenthaler K. Chemical investigations of Plants. G Bell and sons, London; 1930.

115. Sallie R, Tredger JM, Willam R. Drug and the liver. *Biopharm Drug Dispos* 1951; 12: 251-259.

116. Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L *et al.* Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. *N Engl J Med* 2000; 343: 905.

117. Schmidt BM, Ilic N, Poulev A, Raskin I. Toxicological evaluation of a cichory root extract. *Food Chem Toxicol* 2007; 45 :1131-1139.

118. Sell S, Beckar FF. Alpha-feto protein. *Natl Cancer Inst* 1978; 60: 19-26.

119. Shen XD, Song GB, Yan RB *et al*. Research progress of mat and oxymatirne in the anti-tumor mechanism. *J. Chongqing Univ* 2005; (Natural Science Edition) 28: 125-128.

120. Siegers CP and Younes M. Effect of bioflavonoids on lipid peroxidation induced by glutathione depletion. *Pro Int Bioflavonoid Symposium, Munich*, *FRG*, 1981; pp. 409.

121. Sikkim H, Mulee B. Lipid peroxidation and antioxidant enzymes in the blood of rats treated with benzo[a]pyrene. *Chem Biol Interact* 2000; 127: 139-150.

122. Singh JPV, Selvendiran K, Banu SM, Padmavathi R, Sakthisekaran D. Protective role of Apigenin on the status of lipid peroxidation and antioxidant defense against hepatocarcinogenesis in Wistar albino rats. *Phytomedicine* 2004; 11: 309–314.

123. Singleton VL and Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Viticult 1965; 16: 144-158.

124. Sinha AK. Colorimetric assay of catalase. *Anal Biochem* 1972; 47: 389-394.

125. Sivaramakrishnan V, Shilpa PNM, Kumar VRP, Devaraj SN. Attenuation of N-nitrosodiethylamine induced hepatocellular carcinogenesis by a novel flavonol-Morin. *Chem Biol Interact* 2008; 171: 79-88.

126. Skehan P, Storeng R, Scudiero D, Monks A *et al*. New colorimetric cytotoxicity assay for anticancer drug screening, *J. Natl.Cancer Inst.*1990; 82: 1107–1112.

127. Slinkard K, Singleton VL. Total phenol analysis: automation and comparison with manual methods. *Am J Enol Viticult* 1977; 28: 49-55.

128. Soni KB, Lahiri M, Chakradeo P, Bhide SV, Kuttan R. Protective effect of food additives on aflatoxin-induced mutagenicity and hepatocarcinogenicity. *Cancer Lett* 1997; 115:129-133.

129. Sultana S, Perwaiz S, Iqbal M, Athar M. Crude extracts of hepatoprotective plants Solanum nigrum and *cichorium intybus* inhibit free radical –mediated DNA damage. *J. Ethnopharmacol* 1995; 45 : 189-192.

130. Suntar I, Akkol EK, Keles H, Yesilada E *et al.* Comparative evaluation of traditional prescriptions from *Cichorium intybus L*. for woundhealing: Stepwise isolation of an active component by invivo bioassay and its mode of activity . *J. Ethnopharmacol* 2012; 143: 299-309.

131. Szas G. Reaction rate method for gamma glutamyl transferase activity in serum. *Clin Chem* 1976; 22: 2031-2055.

132. Tabassum N, Qazi MA, Shah A, Shah MY. Curative potential of Kashni (Cichorium intybus Linn.) extract against carbon tetrachloride induced hepatocellular damage in rats *Pharmacologyonline* 2010; 2: 971-978.

133. Takagi K, Lee EB. Pharmacological studies on *Platycodon grandiflorum* A. DC. Activities of crude platycodin on respiratory and circulatory systems and its other pharmacological activities. *Yakugaku Zasshi* 1972; 92: 969–973.

134. Takuji T, Toshihiro K, Toshihiko K, Aijin W, Masumi S, Kiyohisa O, Hideki M. Inhibition of 4-nitroquinoline-1-oxide- induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. *Carcinogenesis* 1993; 14 (7): 1321-1325.

135. Tezcan F, Kolayli S, Sahin H, Ulusoy E, Erim BF. Evaluation of organic acid, saccharide composition and antioxidant properties of some authentic Turkish honeys. *J Food Nutr Res* 2011; 50: 33-40.

136. Thorgeirsson SS, Grisham JW. Molecular pathogenesis of human hepatocellular Carcinoma. *Nat Genet* 2002; 31 (4): 339–346.

137. Verna L, Whysner J, Williams GM. *N*-nitrodiethylamine mechanistic data and risk assessment: bioactivation, DNA-adduct formation, mutagenicity, and tumor intiation, *Pharmacol Ther* 1996; 71: 57–81.

138. Vickers A and Zollman C. ABC of complementary medicine: herbal medicine. *BMJ* 1999; 319: 1050 -1053.

139. Wan Xu-Ying, Luo Ming, Li Xiao-dong, He Ping. Hepatoprotective and antihepatocarcinogenic effects of glycyrrhizin and matrine. *Chemico-Biological Intercactions* 2009; 181: 15-19.

140. Wani MC, Taylor HL, Wall ME, Coggon P *et al*. Natural diterpenoid isolated from the stem bark of the Pacific yew tree (*Taxus brevifolia* Nutt.). It promotes the assembly of stable microtubules and inhibits the disassembly process of microtubules to tubulin. *Am Chem Soc* 1971; 93: 2325.

141. Waris G, Ahsan H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J Carcinogen* 2006; 5: 14.

142. Wattenberg LW, Estensen RD. Chemopreventive effects of myo-inositol and dexamethasone on benzo[a]pyrene and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone-induced pulmonary carcinogenesis in female A/J mice. *Cancer Res* 1996; 56: 5132-5135.

143. Waugh A and Grant A. Ross and Wilson's Physiology in health and illness, 11th edition, *Churchill Livingstone, Elsevier*, 2010.

144. Wen YL, Han QL. The pharmacology research progression of the *Sophora flavescens base.* Chin. *J. Pract. Chin. Modern Med* 2006; 19: 473-475.

145. Wooten IDP. Micro-analysis in medical biochemistry, 4th ed, *J and A Churchill Ltd, London,* 1964; p. 138-140.

146. Wroblewski F. The clinical significance of transaminase activities of serum. *Am J Med* 1959; 27: 911-923.

147. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J Agric Food Chem* 1995; 43(1):27- 32.

148. Zafar R, Ali SM. Anti- hepato toxic effects of root and root callus extracts of *cichorium intybus L. J. Ethnopharmacol* 1998; 63: 227-231.

149. Zamule SM, Coslo DM, Chen F, Omiecinski C. Differentiation of human embryonic stem cells along a hepatic lineage. *Chemico-Biological Interactions* 2011; 190 (1): 62-72.

150. Zhang D, Quantick PC, Grigor JM. Changes in phenolic compounds in litchi (*Litchi chinensis* Sonn.) fruit during postharvest storage. *Postharvest Biol Technol* 2000; 19: 165–72.